Molecular analyses of drinking water bacteria critical for human health issues

Distinction between live and dead species and high resolution *in situ* detection of pathogenic bacteria exemplified for *Legionella pneumophila*

Von der Fakultät für Lebenswissenschaften
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
zur Erlangung des Grades einer
Doktorin der Naturwissenschaften
(Dr. rer. nat.)
genehmigte
D i s s e r t a t i o n

von Leila Kathrin Kahlisch
aus Hameln

1.Referent: Privatdozent Dr. Manfred G. Höfle
2.Referent: Professor Dr. Michael Steinert
eingereicht am: 19.05.2010
mündliche Prüfung (Disputation) am: 13.08.2010

Druckjahr 2010

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht.

Publikationen

***Leila Kahlisch**, Karsten Henne, Josefin Draheim, Ingrid Brettar, and Manfred G. Höfle. High-resolution in situ genotyping of *Legionella pneumophila* populations in drinking water by Multiple-Locus Variable-Number of Tandem Repeat Analysis (MLVA) using environmental DNA.
Submitted

Leila Kahlisch, Karsten Henne, Lothar Gröbe, Ingrid Brettar, and Manfred G. Höfle. Assessing the species composition of viable bacteria in drinking water using Fluorescence Activated Cell Sorting (FACS) and community fingerprinting.
Submitted

Leila Kahlisch, Karsten Henne, Lothar Gröbe, Josefin Draheim, Manfred G. Höfle and Ingrid Brettar. Molecular analysis of the bacterial drinking water community with respect to live/dead status.
Water Science & Technology - WST Vol 61 No 1 pp 9–14

Karsten Henne, **Leila Kahlisch**, Josefin Draheim, Ingrid Brettar and Manfred G. Höfle. Polyvalent fingerprint based molecular surveillance methods for drinking water supply systems.
Water Science & Technology: Water Supply - WSTWS Vol 8 No 5 pp 527–532

Tagungsbeiträge

Leila Kahlisch, Karsten Henne, Lothar Gröbe, Josefin Draheim, Ingrid Brettar and Manfred G. Höfle. Community analysis and taxonomic identification of drinking water bacteria with respect to live/dead status. “15th International Symposium on Health-Related Water Microbiology”. Naxos, Griechenland, 2009. (Poster)

Leila Kahlisch, Karsten Henne, Josefin Draheim, Lothar Groebe, Ingrid Brettar, Manfred Höfle. Community analysis and taxonomic identification of drinking water bacteria with respect to live/dead status. “How dead is dead - Survival and final inactivation of microorganisms“ - Symposium der Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM)”. Bochum, Deutschland, 2009.

Leila Kahlisch, Karsten Henne, Ingrid Brettar, Josefin Draheim and Manfred G. Höfle. Dead or alive? - A question of general relevance to aquatic bacteria and of special importance for drinking water. “Jahrestagung der Deutschen Gesellschaft für Limnologie (DGL)”. Konstanz, Deutschland, 2008.

Leila Kahlisch, Karsten Henne, Josefin Draheim, Ingrid Brettar and Manfred G. Höfle. High-resolution *in-situ* analysis of *Legionella pneumophila* populations in drinking water by MLVA-genotyping of environmental DNA. “Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM)”. Hannover, Deutschland, 2010. (Poster)

* zwischenzeitlich veröffentlicht in:
Applied and Environmental Microbiology Vol 76 No 18 pp 6186-95

Wer anderen etwas vorgedacht,
wird jahrelang nur ausgelacht.
Begreift man die Entdeckung endlich,
so nennt sie jeder selbstverständlich.

Wilhelm Busch
(1832-1908)

Meiner Familie und

Dennis

Acknowledgements / Danksagung

Mein herzlichster Dank gilt **PD Dr. Manfred G. Höfle** für die Überlassung des spannenden und interessanten Themas und der guten und andauernden Betreuung während meiner Doktorarbeit.

Vielen Dank an **Prof. Michael Steinert** für die bereitgestellten Legionellen Stämme und die Bereitschaft in meiner Disputation als Prüfer und Korreferent zu fungieren.

Herzlichen Dank für die Übernahme der Drittprüferschaft an **Prof. Dieter Jahn**.

Besonders bedanken muss ich mich an dieser Stelle bei meinem Mitdoktoranden **Karsten Henne**. Ohne ihn wäre ich wohl so manches Mal bei Auswertungen verzweifelt…Und es wäre wahrscheinlich nur 1/65 so lustig im Labor gewesen. Ich möchte ja gar nicht an das Arbeiten im Hochvakuum denken….

Ganz besonderer Dank gilt **Josefin (Josi) Draheim** unserer guten Seele des Labors D0.04, die mir immer mit viel Tatkraft und Hilfe zur Seite gestanden hat und uns so manches Mal mit Waffeln überrascht hat.

Vielen lieben Dank auch an **Dr. Ingrid Brettar** für die Hilfe an den Manuskripten und die vielen hilfreichen Anmerkungen zu meiner Dissertation.

Herzlich bedanken möchte ich mich natürlich auch bei **Julia Strömpl** für Ihre Hilfsbereitschaft im Labor, bei **Rolf Kramer** für die nette Ko-Dokrorandenschaft, bei **Erika Harth-Chu** für die Hilfe mit den Multiplex PCRs und bei **Manuela Hölscher** für ihren extremen Pipettiereinsatz im Dezember 2009.

Danke auch an das D0.17 Labor: **Andreia**, **Marcela**, **Maira**, **Esther** und **Susanne**! Es war immer lustig mit Euch! Besonders liebe Grüße an dieser Stelle an Frau **Jennifer Knaak** – die mit mir nicht nur den gleichen Humor teilt…wir haben auch die gleiche Uhr, ne?

Vielen Dank auch an das liebe Sequenzer Team, **Annette** und **Michaela**. Danke, dass ihr meine Sequenzen immer schön fleißig schnell bearbeitet habt! Ihr habt dazu beigetragen, dass meine Arbeit (fast) pünktlich fertig wurde. Herzlicher Dank auch an **Birgit**. Sie war die erste die mich am HZI empfing und hat mich sogleich gut integriert.

Danke auch an all die anderen, die mich während meiner Doktorarbeit in irgendeiner Weise begleitet haben, vor allem an **Iris, Melissa** und **Silke** für die vielen netten Mittagspausen! Der kompletten **VAC** Gruppe danke ich natürlich auch. Vor allem meine Büro-Kolleginnen und Kollegen **Sebastian**, **Kirsten**, **Rimma**, **Beata**, **Peggy** und **Miriam**.

Mein aufrichtiger Dank geht an meine Eltern **Waltraud** und **Yehia**. Ihr habt mich bis heute bei allem, was ich getan habe und tun möchte, in jeder erdenklicher Weise unterstützt. Ich werde euch auf immer dankbar sein.

Meiner Schwester **Nadia** danke ich für ihre aufmunternden Worte wenn es mir mal nicht so gut ging, ihren unvergleichlichen Humor und die ehrliche Freundschaft zu mir.

Ich danke meinem Ehemann **Dennis**, der mit mir durch dick und dünn geht, mich jeden Tag zum Lachen bringt und der meinem Leben einen neuen Sinn gegeben hat. Ich liebe dich mein Schatz!

Summary

The transmission of pathogens by drinking water can pose a significant health risk to the consumer. Although the water treatment aims at eliminating or killing the bacteria a highly diverse bacterial microflora is present in the finished drinking water reaching the consumers tap. Today's tools to assess hygienic quality and potential health risks of drinking water are still mostly cultivation based. The drawbacks of these methods (e.g. bacteria that enter a viable but nonculturable state) confirm the need for new detection tools in drinking water surveillance. Molecular tools that are based on the analysis of nucleic acids (DNA and RNA) can therefore help to detect and quantify microbial pathogens in drinking water and to analyze all bacteria in a given drinking water sample.

In this study, molecular and cellular tools have been used to assess the community structure and viability of the drinking water microflora of a small scale drinking water distribution system located in Braunschweig, Germany. During one and a half year, water from the tap was sampled and analyzed to understand seasonal dynamics affecting the overall community structure of drinking water. The analyses included SSCP fingerprinting, sequencing of relevant bands and phylogenetic assignment of the 16S rRNA sequences. In the following, live/dead staining and fluorescence activated cell sorting (FACS) were used to assess the viable and the dead part of the drinking water microflora. The developed approach enabled monitoring of the bacterial drinking water community and assessment of the physiological state of taxonomic groups of interest. Applied on a time to time basis, the approach can therefore contribute to the development of a more efficient and safer drinking water treatment.

The emergence of so called "new pathogens" has become a considerable problem for drinking water production and distribution. These pathogens are often environmental bacteria that find their way into our water distribution system, where they can survive and grow. Since species of the genus *Legionella* are ubiquitous in many natural freshwater environments it is nearly impossible to prevent their entry into man-made aquatic environments. They occur in most drinking water supply systems (DWSS) and pose a significant health threat in case the most infectious species and serotype, e. g. *Legionella pneumophila* serogroup 1, is present. Epidemiological analyses of infections caused by *L. pneumophila* depend on the accurate identification of strains, preferably at the clonal level. Available genotyping methods (like MLST and MLVA) require a prior isolation of *L. pneumophila* from the environmental or clinical sample. In this study, the approved MLVA-8 typing scheme for *L. pneumophila* was adapted to environmental DNA for a fast and accurate typing of *L. pneumophila* isolates in one environmental samples without the need for cultivation based methods. We succeeded in amplifying the different loci and separated the amplicons on SSCP gels. The approach enabled not only the detection of different *L. pneumophila* genotypes in environmental samples but also the possibility for sequencing of the VNTR products from the SSCP gel. This new method is also transferable to the typing of other pathogens of interest and therefore presents a promising new tool for molecular epidemiology of pathogens *in situ*. Both developed approaches allow a better assessment of risks associated with bacteria in drinking water critical for human health and support its improved processing and management.

Zusammenfassung

Die Übertragung mikrobieller Krankheitserreger über das Trinkwasser stellt für den Verbraucher ein erhebliches Gesundheitsrisiko dar. Obwohl Wasseraufbereitungsmassnahmen darauf abzielen, Bakterien im Trinkwasser gezielt zu töten oder zu beseitigen, enthält das fertige Trinkwasser aus dem Hahn eine hoch diverse bakterielle Mikroflora. Die Werkzeuge, um die hygienische Qualität von Trinkwasser zu bewerten und das potentielle Gesundheitsrisiko einzuschätzen, sind allerdings oft noch kultivierungsabhängig. Die vielen Nachteile dieser Methoden (z.B. Bakterien im VBNC status) bestätigen die Notwendigkeit für neue Detektionswerkzeuge in der Trinkwasserüberwachung. Molekulare, nukleinsäurebasierte Methoden, können dabei helfen, mikrobielle Krankheitserreger im Trinkwasser zu detektieren, zu quantifizieren und bakterielle Gemeinschaften besser zu charakterisieren.

In dieser Studie wurden zelluläre und molekulare Methoden eingesetzt, um die Struktur und den Anteil lebender Zellen der Trinkwassermikroflora eines kleinen Trinkwasserversorgungssystemes in Braunschweig zu untersuchen. Anderthalb Jahre lang wurden in regelmäßigen Abständen Wasserproben entnommen und analysiert, um die saisonale Dynamik die die Struktur der Gemeinschaft beeinflusst besser zu verstehen. Die Analysen beinhalteten SSCP Fingerprinting, Sequenzierung von wichtigen SSCP Banden und die phylogenetische Zuordnung der erhaltenen 16S rRNA Sequenzen. Im Folgenden wurde eine lebend/tot Färbung mir einer Fluoreszenz aktivierten Zellsortierung (FACS) kombiniert, um den Anteil der lebenden und der der toten Bakterien der Trinkwassermikroflora zu bestimmen. Der entwickelte Ansatz ermöglicht das Monitoring der bakteriellen Gemeinschaft des Trinkwassers und die Einschätzung des physiologischen Zustandes von taxonomisch wichtigen Gruppen. Daher kann der entwickelte Ansatz als ein wertvoller Beitrag zur Entwicklung einer effizienteren und sichereren Trinkwasseraufbereitung gesehen werden.

Das Auftreten sogenannter „neuer Pathogene“ ist zu einem bedeutenden Problem bei der Produktion und der Verteilung von Trinkwasser geworden. Diese Erreger sind oftmals Bakterien aus der Umwelt, die den Weg in unsere Trinkwasserverteilungssysteme finden und dort Bedingungen vorfinden, die ihnen das Überleben und oft sogar ein Wachstum ermöglichen. Da Spezies des Genus *Legionella* ubiquitär in natürlichen Süßwasser-Habitaten vorkommen ist es nahezu unmöglich, ein Eindringen in künstlich geschaffene Wassersysteme zu verhindern. Daher kommen Legionellen in nahezu jedem Trinkwasserversorgungssystem vor und können ein erhebliches Gesundheitsrisiko darstellen, vor allem wenn sie in der infektiösesten Form auftreten (*Legionella pneumophila* Serogruppe 1). Epidemiologische Analysen von Infektionen, die durch Legionellen ausgelöst werden, benötigen die genaue Identifizierung von Stämmen, möglichst bis zur klonalen Stufe. Zur Verfügung stehende Genotypisierungsmethoden (z.B. MLST und MLVA) setzen eine Isolierung von, *L. pneumophila* aus der Umwelt- oder klinischen Probe voraus. In dieser Arbeit wurde das anerkannte MLVA-8 Genotypisierungsschema für *L. pneumophila* Isolate verbessert und weiterentwickelt, um ohne vorhergehende Isolierung eine Genotypisierung von *L. pneumophila* in Umweltproben zu ermöglichen. Dafür wurden die einzelnen VNTR loci über eine PCR vervielfältigt und die entstandenen Produkte auf SSCP Gelen aufgetrennt. Der Ansatz ermöglichte hierbei nicht nur die Detektion von verschiedenen *L. pneumophila* Genotypen in einer Umweltprobe sondern zudem die Möglichkeit der direkten Sequenzierung der Produkte aus den SSCP Gelen. Diese neuartige Methode kann für eine Vielzahl anderer Pathogene und Probenmaterialien eingesetzt werden und stellt daher ein neues vielversprechendes Werkzeug für die molekulare Epidemiologie von Pathogenen *in situ* dar. Beide in dieser Arbeit entwickelten Ansätze erlauben eine bessere Einschätzung von Risiken, die durch Bakterien im Trinkwasser hervorgerufen sein können. Sie stützen somit eine verbesserte Trinkwasseraufbereitung und ein verbessertes Trinkwassermanagement.

Table of contents

CHAPTER 1

General introduction

Leila Kahlisch

Dept. Vaccinology and Applied Microbiology
Helmholtz Center for Infection Research (HZI)
Inhoffenstrasse 7, 38124 Braunschweig, Germany

Chapter 1 General introduction

Transmission of pathogens by drinking water can be a significant cause of illness for the world's population. According to the World Health Organization (WHO), 3.4 million deaths per year are water related and among these 2.2 millions are caused by diarrhoeal disease affecting mostly small children and elderly persons (WHO/Unicef 2000 / (59)). Especially in developing countries, outbreaks of waterborne diseases are often caused by insufficient sanitation of human and animal waste or unprotected wells. But the problem is not only limited to developing countries. Although people in industrialised countries have constant access to high quality public water supply systems, health risks by waterborne pathogens may occur through technological failures, mismanagement of fresh-water resources and/or inappropriate detection methods. Broadening our knowledge of pathogens and their pathogenesis in drinking water can help to improve water treatment measures and drinking water safety in general. This process should ideally include tools for the precise detection, identification and quantification of all three types of microorganisms: viruses, bacteria and protozoa. Additionally, factors influencing the virulence of pathogens should be further characterized (e.g., environmental conditions and water treatment can have major effects). Furthermore, we have to elucidate the complex process of infection by drinking water pathogens which depends e.g. on the type of pathogen, the infectious dose and also the immune status of the exposed person. For example, some bacteria like *Mycobacterium avium* can pose a significant health risk to immunocompromised persons (particularly AIDS patients (7)) but are of minor relevance for a healthy immunocompetent person.

1.1 The detection gap for pathogens in drinking water

Today's tools to assess hygienic quality and potential health risks of drinking water are still cultivation based. Due to several critical constraints these methods used so far can be termed inappropriate. First of all, the cultivation of indicator species like *Escherichia coli* on standard plate count media does not represent the majority of bacteria present in drinking water comprising a variety of mostly heterotrophic species. Furthermore, *E. coli* and enterococci are much less chlorine resistant than other bacteria or even cysts and oocysts of emerging pathogens in drinking water like *Giardia* sp. and *Cryptosporydium* sp.. Additionally, freshwater bacteria can enter a viable but nonculturable (VBNC) state thus complicating the detection restricted to culture dependent methods (76). Besides, some bacterial pathogens like legionellae live mostly intracellularly in their natural hosts (fresh water species of amoebae) or in biofilms, where they are protected against disinfection measures or other adverse conditions. Together, these drawbacks confirm the need for new detection tools in drinking water surveillance. In the last two decades, new molecular detection methods

gained more importance enabling a faster and more accurate identification and quantification of bacterial pathogens in drinking water. For an implementation of these methods for modern water surveillance, some relevant questions have to be answered: 1) Which microorganism should be detected? 2) Which level of taxonomic resolution is requested? 3) What is the detection limit of the assay? and 4) Which modern equipment is needed and what are the analysis costs per sample? The last point is of special importance for developing countries, where these technologies should be affordable and achievable with respect to laboratory facilities and staff.

1.2 Molecular tools for the analysis of microbial communities and pathogens in drinking water

Plate counts have been and still are a tool for studying bacteria in environmental samples. It is well known that in most habitats (e.g. freshwater (48), soil or sediments (48, 101)) direct microscopic counts exceed the number of plate counts by several orders of magnitude. This phenomenon was observed by microbiologists for considerable time and was called “the great plate count anomaly” by Staley and Konopka (95). It is widely accepted that the majority of cells that are microscopically detected can be viable but do not form visible colonies on a defined culture medium (86). One major reason for this high amount of non-cultivable cells is that the applied cultivation methods are just not suitable or microorganisms have entered a viable but nonculturable (VBNC) state (23, 46). VBNC was first used in 1982, when Colwell et al. found that cells of *E. coli* and *V. cholerae* can be present in a “nonrecoverable stage of existence, but remain viable” (111). In this state, metabolic activity of cells is typically low while they are unable to form visible colonies on culture media (77). A VBNC state is known for a large number of species including human pathogens such as *Campylobacter spp., Mycobacterium tuberculosis, E. coli* (including EHEC strains) and *Legionella pneumophila* (for an overview of bacteria able to enter the VBNC state see J.D. Oliver 2005 (77)). Although the importance of these nonculturable bacterial cells for infection is not yet fully understood, it is known that these cells could retain their virulence and should be considered as potentially hazardous. Because of these important drawbacks of culture dependent methods, they can be termed insufficient for studying microbial community structure and compositions. During the last two decades, molecular methods were developed that can complete or replace the culture based methods.

The first attempts to characterize environmental samples by molecular methods were made in the middle of the 1980s. At this time, 5S rRNA molecules were directly extracted from a mixed sample, electrophoretically separated and phylogenetic trees were constructed based on obtained sequence information (94). Although the information content of the 120 nucleotide 5S rRNA molecule was relatively small and only less complex ecosystems could

have been investigated, these first studies yielded interesting phylogenetic information. In 1986, Olsen et al. (78) recommended the use of the larger 16S rRNA molecule. The first application of the relatively laborious procedure of cloning and sequencing was achieved in 1990 by Schmidt et al. analysing an oligotrophic marine community (91). Besides numerous unknown sequences, fifteen unique bacterial sequences were obtained in the study (including *Cyanobacteria* and *Proteobacteria*). With the advent of PCR (88), it became possible to amplify 16S rRNA gene fragments directly from a mixed DNA sample and to construct gene libraries. Giovannoni et al. (36) were the first to analyze clone libraries of 16S rRNA genes amplified from the Sargasso Sea (North Atlantic) picoplankton by PCR methods. Based on the retrieved rRNA genes, they identified a new planktonic alpha-proteobacterial clade (SAR 11) which is now estimated to make up one half of the microbial community in Atlantic surface waters (69). Since then, sequence analysis of the 16S rRNA gene has been widely used to perform taxonomic studies (20, 80, 90) and is a useful tool for the detection of novel uncultured strains and pathogens.

For drinking water, community analyses, detection and quantification of microbial pathogens can be done on the basis of nucleic acids. One major advantage of detection assays using nucleic acids is that they can be stored for a long-time through preservation by freezing. A possible scheme for the work flow of a nucleic acid based analysis of drinking water is shown in Fig. 1. In the beginning, the microorganisms of a given water sample are harvested. This can be done either by filtration on a filter with a pore size retaining bacteria (suitable for small amounts of samples from one to five litres, depending on turbidity and used filter diameter) or by fractionated ultrafiltration (up to hundreds of litres) which may also retain viruses if the proper material and cut-off value is selected. These harvested biomasses can now be stored at -20℃ or -80℃ until further a nalyses can be proceeded. This is an essential advantage over cultivation based methods which require fresh live material and a fast sample processing. Therefore, the method is also suitable for sampling in the field, requiring only the possibilities for sampling and freezing of the samples.

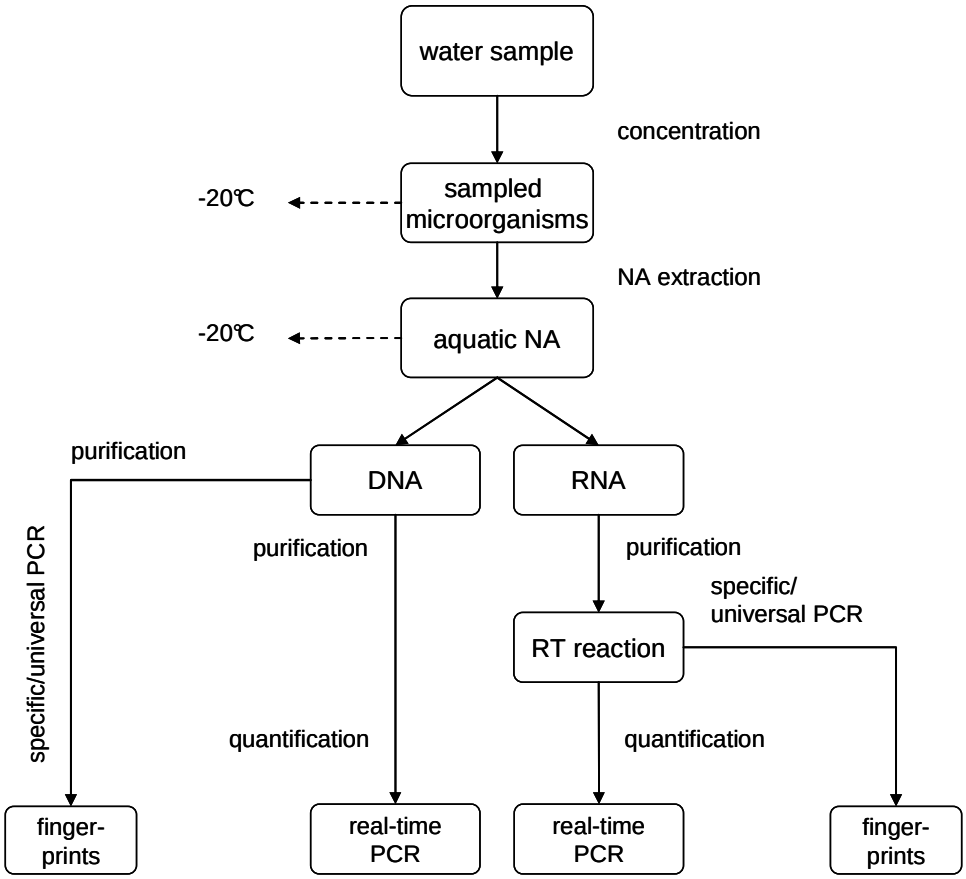


Fig. 1. Scheme for major steps in the molecular analysis of drinking water. RT = reverse transcription; NA = nucleic acids. Dashed lines indicate possible specimen storage (modified from (17)).

Frozen biomasses (e.g. on filters) are processed by appropriate nucleic acid extraction methods, which are normally based on the lysis of the cells followed by phenol/chloroform extraction chemistry and/or precipitation of nucleic acids by alcohol in the presence of chaotropic salts (e.g. guanidinium thiocyanate). DNA and RNA can be extracted separately or simultaneously from the same biomass (27) and stored frozen until further analysis. For the following steps in molecular analysis, a purification step should be included to clean the environmental nucleic acids from substances inhibiting further analyses (e.g. humic acids, high amounts of salts). This can be done by commercially available silica filter columns or another precipitation step. Owing to the normally DNA-based molecular analyses, environmental RNA has to be transcribed into DNA by a reverse transcriptase step, usually performed by an RNA-dependent DNA polymerase isolated from viruses. Now, either a molecular detection of a targeted pathogen can be conducted (e.g. by specific PCR-based assays) or a universal PCR followed by a suitable fingerprint technique to separate the products. The generation/selection of primers has to correspond to the level of taxonomic resolution which should be achieved. 16S rRNA gene targeted general bacterial primers for example enable the analysis of a complex microbial community whereas *Legionella*

pneumophila specific primers will only amplify the most important species of the genus *Legionella* (i.e. *L. pneumophila*) (4). To use a specific primer pair also for quantification by real-time PCR, a molecular standard for the targeted pathogen is necessary. For example, extracted DNA of the genome-sequenced *Legionella pneumophila* Philadelphia strain can be used as a standard in real-time assays to quantify this pathogen in water samples (99).

All molecular techniques require to a great extent standardization and validation measurements in order to prevent false over- or underestimations of pathogens detected in drinking water. Contaminations are a critical issue for these methods. Though some of the analyses are too complex and also too expensive to be used in routine drinking water surveillance, the detection and quantification of pathogens with the help of molecular detection tools can contribute to a better understanding of their appearance and spread and therefore lead to an improved management and quality control for drinking water processing.

1.3 Overall community structure of the drinking water microflora

Microorganisms present in drinking water usually derive from natural freshwater sources like ground water, lakes or rivers (28). A bacterial group can be termed “typical for freshwater”, if they were shown to occur in several freshwater habitats and if databases for 16S rRNA gene sequences contain more sequences from freshwater sources than from other sources (for example marine or terrestrial environments) (62). Typical bacterial groups occurring in freshwater are *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* as well as members of the phyla *Actinobacteria, Verrumicrobia* and *Cyanobacteria* (38, 113). These groups have also been identified in a previous study of the drinking water supply system analysed in the present work (28). In a study of 15 diverse lakes from northern Europe, it was shown, that the distribution and variation of the bacterial taxa were closely associated with environmental factors such as pH, temperature, and the retention time of the lakes (62). Changes in the community structure of the freshwater microflora can occur due to natural effects like floods or storms but can also be caused by pollution (e.g. run-off containing manure). As shown previously, drinking water deriving from natural freshwater is strongly influenced by variations occurring in the source water (for example seasonal variations) (28, 43).

For the characterization of bacterial communities, different approaches are available. Besides community fingerprints that have been extensively used for the analysis of freshwater habitats like lakes (61), fluorescence in situ hybridization (FISH) can provide additional information about cell number and shape and allows quantification (64). For drinking water, where most cells can not be cultured and a large fraction of the detected species have been assigned to uncultured groups, methods not requiring cultivation (like PCR) have become valuable tools for the analysis of microbial communities (28, 113).

1.4 Assessing the viability of microorganisms in drinking water

Many people believe that drinking water from their tap is free of microorganisms. If we count the microorganisms in one litre of drinking water by epifluorescence microscopy we know that this is not the case - in contrast, the number of cells per litre is around 10^8 (see for example (81)). And despite the water treatment process aims at eliminating or killing the bacteria, drinking water shows a highly diverse microflora, partially due to regrowth after the treatment process (17). This microflora is composed of bacteria originating from the source water, bacteria that have grown in bulk water and the biofilm of the distribution network. Often oxidizing agents, such as ozone, chlorine or chloramine, are applied to control occurrence and regrowth of bacteria in the distribution system. However, oxidizing agents have been found to split organic compounds into organic acids accessible for heterotrophic bacteria (56) and it is suggested that this easily assimilable organic carbon (AOC) is the main cause for microbial regrowth in drinking water distribution systems (55, 58, 63). This also contributes to the growth of pathogenic bacteria in bulk water and biofilms and can pose a potential risk for the end user (102). In this regard, it is crucial to determine the physiological status of the present bacterial species. The implementation of new molecular tools on the basis of nucleic acids enabled the detection of both, culturable and nonculturable (VBNC) bacteria. Since DNA and also stable ribosomal RNAs can persist in the environment long after the bacterium has lost its viability, molecular methods based on the PCR amplification of the 16S rRNA gene do not provide an estimate of viable cells in a bacterial community (50, 73).

A method for the determination of viable cells can be the detection of rapidly degrading RNA (mRNA) instead of the rather stable DNA or RNA molecules. Due to the rapid turnover the use of mRNA via reverse transcription-PCR (RT-PCR) targeting suitable genes seemed highly indicative for viable cells (54). Nevertheless, working with unstable RNA molecules is laborious and contaminating RNA-degrading enzymes that are ubiquitously present in the environment can lead to severe problems.

Since the broadly applied molecular methods based on 16S rRNA gene analyses (28) lack the possibility to assess the viability of single members of the bacterial community, parameters that can be linked to cell viability have been extensively investigated in the last decade. These viability measurements range from detection of specific metabolic activities to cell components (related to viability). For example, the measurement of DNA content gave a good correlation to viability in natural planktonic bacteria (35), where bacteria with apparent high DNA (HDNA) content are differentiated from those having an apparent low DNA content (LDNA). Additionally, the ATP content of a cell could also provide some insights into its physiological status (11).

In the last years, the application of fluorescent dyes helped to complement existing molecular tools by giving insights into the physiological state of single members of the bacterial community. A broad range of “viability stains” that can be used for microscopical distinction of cells are available on the market all having different targets of the cell or its metabolism (see Fig. 2) (11). Membrane potential (MP) for example, plays a central role in different cellular processes (e.g. ATP synthesis, transport, regulation of intracellular pH, etc.). To measure MP in bacteria, voltage-sensitive dyes like the anionic lipophilic oxonol $DiBAC_4(3)$ have been developed (see Fig. 2). For this assay, a pre-treatment with EDTA is necessary to allow the lipophilic $DiBAC_4(3)$ to penetrate the outer membrane (71). Because of this permeabilization step, which varies strongly depending on the species, this method is not generally applicable to natural and complex samples like drinking water. The measurement of enzyme activity, e.g. dehydrogenase activity can also be used as a viability indicator because these processes are normally directly related to the energy metabolism in respiring cells. Based on this principle, the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is used to quantify metabolically active bacteria under both aerobic and anaerobic conditions (12, 93). However, dyes measuring enzymatic activities can have cytotoxic effects thus leading to an underestimation of the metabolically active fraction of the cells (103). Some dyes, like ethidium bromide or carboxyfluoroscein, are known to be actively removed by efflux pumps from the cell, means probe efflux is used as a measure for cell activity (71). Since this technique has only been validated for a few species in culture it is not yet considered suitable for the analysis of environmental samples.

The measurement of membrane integrity is considered as a suitable criterium for cell viability. Due to the various functions that are linked to the plasma membrane (permeability barrier, transport, respiratory activity, etc.), membrane integrity can be considered as crucial for the viability of the cell. Up to now, it is generally assumed that membrane-injured bacteria can be considered as dead (11, 44, 51) although it is still under debate if a bacterium with an injured membrane is still able to recover or not (19). Most of the assays targeting membrane integrity use fluorescent nucleic acid stains, owing to the high concentrations of nucleic acids present within the cells. The two nucleic acid dyes, Propidium Iodide (PI) and SYTO9, can be used to distinguish membrane intact from membrane injured cells (14). SYTO9 (green fluorescence), can pass the cytoplasmic membrane of all cells, whereas propidium iodide (PI, red fluorescence) is only able to enter a cell when the cytoplasmic membrane is damaged (14). Membrane intact cells appear as green, but if the cell membrane is damaged, the presence of both stains results in a red fluorescence.

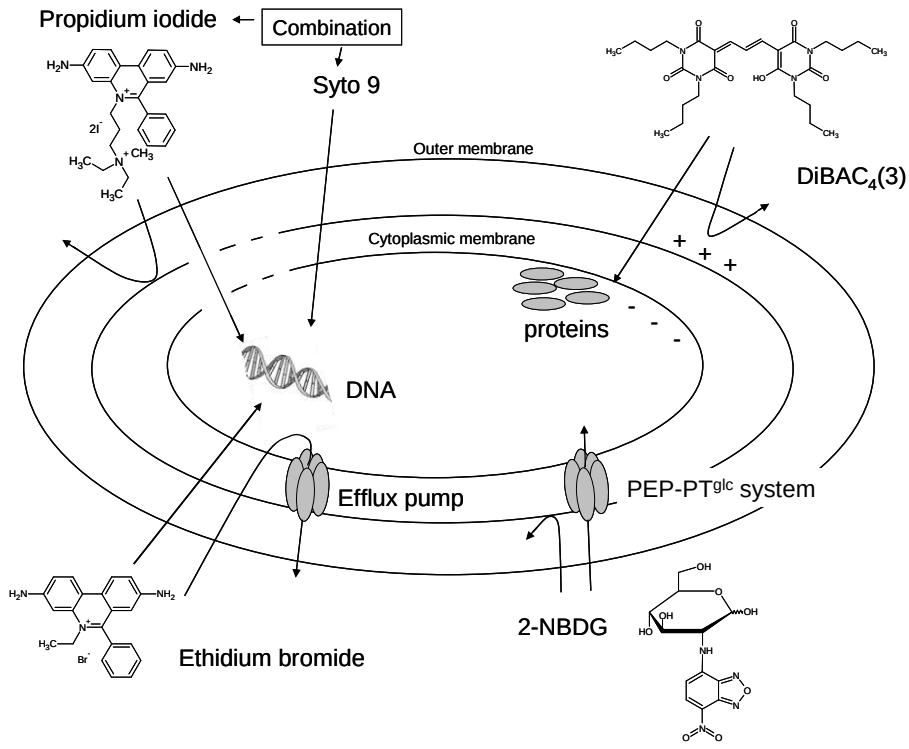


Fig. 2. Viability indicators (fluorescence stains) that can be applied in combination with flow cytometry and their function in a gram-negative bacterium (simplified). Functions: Syto 9 (green fluorescence) for total counts; propidium iodide (red) for membrane integrity; ethidium bromide (red) for efflux pump activity; 2-NBDG (green) for glucose uptake activity (PEP-PTglc, the PTS component for glucose transport); DiBAC4(3) for membrane potential. Syto 9 and propidium iodide are commercially available in the BacLight™ Kit (modified from (11)).

This staining procedure has been evaluated and compared by a set of studies to other staining procedures for assessment of the physiological status of bacteria (14, 24, 51). The detected membrane injury was evaluated as a major criterion for cell death where recovery is highly unlikely (10, 24, 51). Together with flow cytometry, the combined use of SYTO9 and PI allows sorting of membrane injured cells from membrane intact cells thus giving the possibility to analyze the two fractions of cells.

Very recently, ethidium monoazide (EMA) was implemented to assess viable cells in complex samples (87). The stain was linked with real-time PCR (EMA-PCR) thus combining the use of a live-dead staining with the advantages of real-time PCR (see Fig. 3). Although EMA has been widely used to differentiate between live and dead cells, the general application of the dye is hampered because the chemical is known to penetrate also life cells of some bacterial species. Though transport pumps can actively export EMA out of metabolically active cells, the remaining EMA can lead to substantial loss of DNA (74). Propidium monoazide (PMA) which is, like Propidium Iodide, highly selective for the detection of only “dead” cells, is thought to be a better choice because of its higher charge leading to a higher impermeability through intact cell membranes (74).

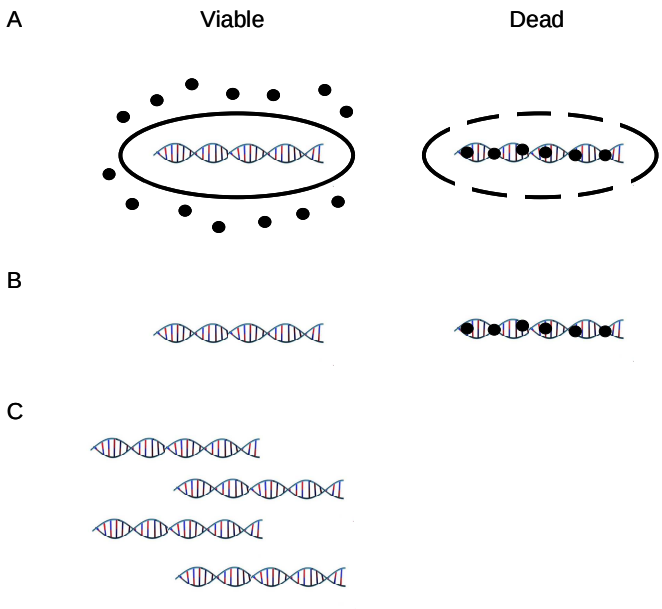


Fig. 3. Principle of viability PCR using EMA/PMA. A: Ethidium or Propidium monoazide is added to the sample containing a mixture of viable and dead cells. The dye can only penetrate dead cells and binds to the DNA molecule. A light exposure of 1min leads to covalent binding and therefore an inactivation of EMA/PMA. B: The DNA fraction from the viable cells remains unstained while the DNA from the dead cells is covalently bound to EMA/PMA. C: During PCR, the DNA not bound to EMA/PMA can be amplified while the DNA from dead cells with bound EMA/PMA cannot be amplified. Figure modified from (87).

Fluorescent dyes currently provide a good tool to distinguish between live and dead microbial cells and can therefore be seen as a substantial improvement over culture-dependent methods. Now researchers are not only able to assess the presence of a bacterium but also its viability. This is of general ecological relevance and of special importance regarding drinking water where pathogenic bacteria can be transmitted to the consumers. The insights gained from these “live/dead” analyses can therefore support measures to improve water treatment and drinking water safety in general.

1.5 Emerging pathogens in drinking water

The emergence of so called “new pathogens” in drinking water is perceived as a problem for drinking water production and distribution (see also Tab. 1). This includes, on the one hand, pathogens that were only recently recognized as pathogens, like *Legionella* spp., *Pseudomonas aeruginosa* or *Mycobacterium* spp. These pathogens are often environmental bacteria that find their way into our water distribution system, where they encounter good conditions for growth and survival. Especially, biofilms and dead ends of the distribution system are niches where they are protected from adverse conditions. On the other hand, well-known pathogens like *Campylobacter* spp. or toxigenic species of *Escherichia coli,* can be transferred from contaminations of the source water and can pose a significant health risk.

Tab. 1. Pathogens in drinking water: Infectious dose, estimated incidence through consumption of drinking water in the United States, survival in drinking water, and potential survival strategies.

Abbreviations: ?, unknown; IC, intracellular survival and/or growth; VBNC, viable but not culturable (adapted from (31)). [b]Infectious dose is number of infectious agents that produce symptoms in 50% of tested volunteers. [c]U.S. point estimates. [d]Very few outbreaks of cholera occur in the United States, and these are usually attributable to imported foods. [e]Data from Breiman and Butler (16). [f]Includes Norwalk virus, poliovirus, coxsachievirus, echovirus, reovirus, adenovirus, HAV, HEV, rotavirus, SRSV, astrovirus, coronavirus, calicivirus, and unknown viruses. [g]Estimated for HAV, Norwalk virus, and rotavirus (107).

		Infectious dose[b]	Estimated incidence[c]	Survival in drinking water (days)	Survival strategies[d]
Bacteria					
	Vibrio cholerae	10^8	(very few)[d]	30	VBNC, IC
	Salmonella spp.	10^{6-7}	59.000	60-90	VBNC, IC
	Shigella spp.	10^2	35.000	30	VBNC, IC
	Toxigenic *Escherichia coli*	10^{2-9}	150.000	90	VBNC, IC
	Campylobacter spp.	10^6	320.000	7	VBNC, IC
	Leptospira spp.	3	?[f]	?	?
	Francisella tularensis	10	?	?	?
	Yersinia enterolitica	10^9	?	90	?
	Aeromonas spp.	10^8	?	90	?
	Helicobacter pylori	?	High	?	?
	Legionella pneumophila	>10	13.000[e]	Long	VBNC, IC
	Mycobacterium avium	?	?	Long	IC
Protozoa					
	Giardia lambia	1-10	260.000	25	Cyst
	Cryptosporidium parvum	1-30	420.000	?	Oocyst
	Acanthamoeba spp.	?	?	?	Cyst
Viruses[f]					
	Total estimates	1-10	6.500.000	5-27[g]	Adsorption/ absorption

The reasons for the emergence of these new pathogens are diverse and yet not fully understood. Some of them were simply not detected due to a lack of detection methods. *Legionella* spp., for example was not detected for a long time because it is not able to grow on standard plate media. Other pathogens, like animal pathogens, have not been associated with drinking water and were hitherto neglected. Additionally, changes in human life style, e.g. the increased use of heated drinking water (requiring warm water provision and storage units) promote the emergence and persistence of new pathogens. *Legionella* spp. and other environmental bacteria are able to use these devices as new habitats.

Even if a pathogen is present in low numbers, it can pose a health risk for the today's growing amount of susceptible people. This includes immunocompromised persons like AIDS patients, patients receiving chemotherapy or organ transplants and elderly people with an age-related compromised immune system. These persons can be subjected to infections which normally do not occur in healthy adults with a good immune status. The complete removal of all pathogens is nevertheless not always possible or affordable. Therefore, recent guidelines like the European Union Council Directive 98/83/EC and WHO guidelines prescribe that drinking water should contain these microorganisms only in such low numbers that the risk for waterborne infections is relatively low and acceptable. These requirements can only be fulfilled if water resources are well protected (for example against fecal contaminations) and an accurate quality control of the water treatment process is implemented.

1.6 *Legionella pneumophila* - a natural waterborne bacterium becomes an emerging pathogen

Legionella pneumophila is a gram-negative, aerobic, monopolarly flagellated rod that is commonly found in natural freshwater environments. In the environment, the bacterium persists and replicates in freshwater protozoa like amoeba. *L. pneumophila* can be transmitted to humans via small droplets of water, e.g. aerosols from cooling towers, shower heads or air conditioning systems. By inhaling those droplets, the bacteria enter the human respiratory tract where they can invade and replicate in alveolar macrophages, causing severe pneumonia (Legionnaires disease, see Fig. 3). Besides this acute form of disease, they are also able to provoke a milder form of respiratory illness which resembles acute influenza, the Pontiac fever (37). *L. pneumophila* was first recognised as human pathogen in 1976 at an outbreak of acute pneumonia occurring among veterans attending a convention in a hotel in Philadelphia, USA (32).

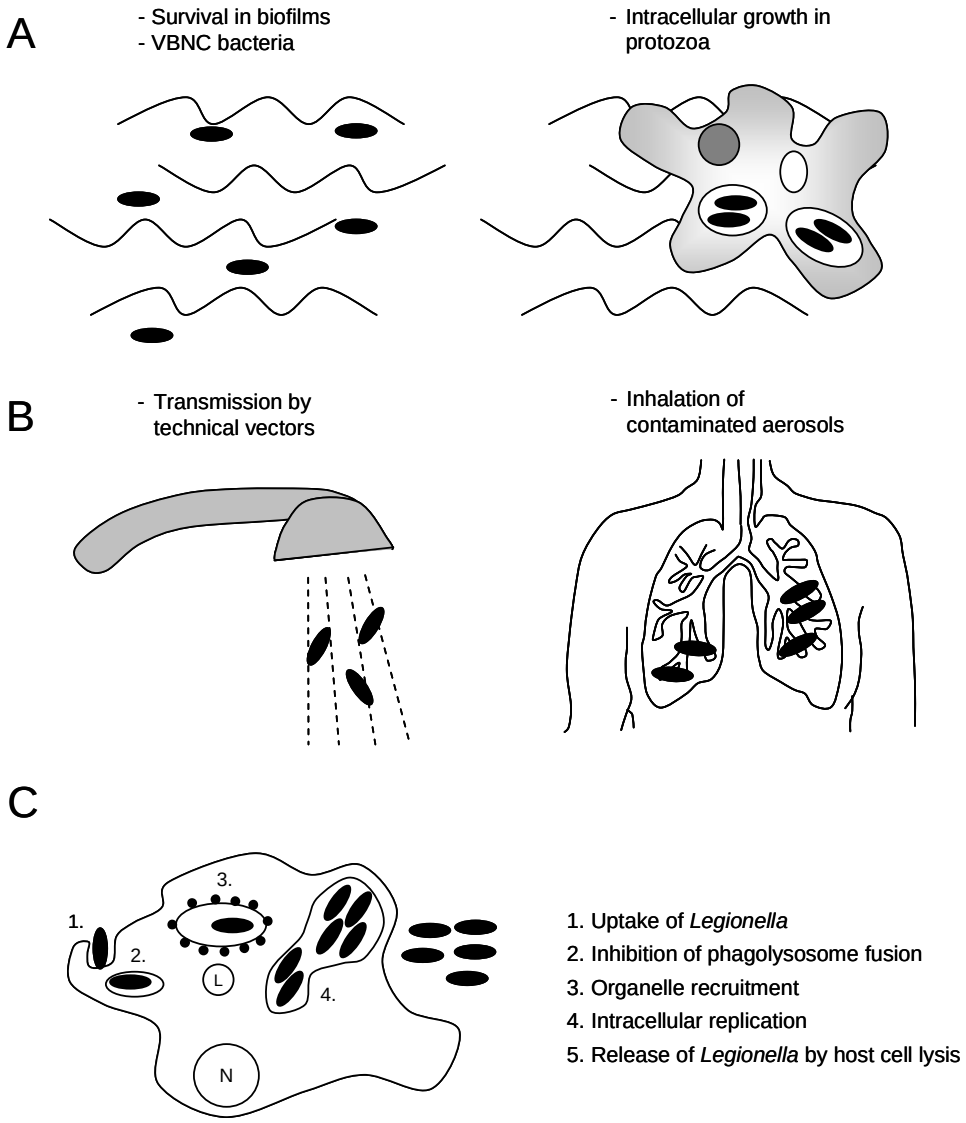


Fig. 3. Infection route of *L. pneumophila* (figure modified from (96)). Abbrevations: N, nucleus, L, lysosome. A: In the environment, *L. pneumophila* replicates intracellularly in protozoa or persists in biofilms. It is also able to enter a viable but nonculturable (VBNC) state. B (infection): By transmission through technical vectors (e.g. aerosols from showerheads or air conditioning systems) *Legionella* can enter the human respiratory tract and invade lung macrophages. C: Upon uptake in alveolar macrophages, *Legionella* inhibits phagolysosomal fusion and replicates within a maturation blocked vacuole. By host cell lysis *Legionella* will be finally released in the environment again.

Though 48 species of the genus *Legionella* (2, 8, 65, 66) exist, *L. pneumophila* is the main pathogen responsible for approximately 91% of all reported community acquired cases of legionellosis shown by an international survey (112). The survey also showed that among the 15 serogroups of *L. pneumophila*, serogroup 1 accounted for 84% of all confirmed cases (112). Despite this fact, it is likely, that many legionellae can cause disease in humans under the appropriate conditions (29). In Europe, a total of 10,322 cases of Legionnaires disease were reported during the years 2000-2002, with infection rates ranging from 0 to 34.1 cases

per million population (49). It can be assumed, that the number of cases is much higher because of the difficulties to distinguish *Legionella* caused disease from other forms of pneumonia. The commonly used detection by urinary antigen test may also contribute to the fact that milder forms of disease may remain undetected because the sensitivity of the test strongly depends on the severity of the disease (13). Additionally, disease that is caused by other *Legionella* species (e.g. *L. longbeachae* is the major cause of legionellosis in Austalia and New Zealand (112)) will not be detected with common antigen tests (70).

Legionella associated disease has emerged upon alteration in human life style in the last half of the 20th century. Mainly the use of hot water, showers and spas, but also the extensive use of air conditioning systems accounted for the manifestation of *Legionella* in man-made environments. Since the pathogen is so ubiquitous in natural aquatic environments, it is nearly impossible to prevent its entry into man-made aquatic systems (96). In drinking water supply systems (DWSS) for example, legionellae can survive in dead-end tubings, stagnated water in plumbings, seldom used facilities (3) or drinking water biofilms (92). Most frequently, the bacterium can be isolated from warm water systems (3). Surveys have shown that legionellae can be detected in 40% of freshwater environments by culture and in up to 80% by PCR-based methods (30). Additionally, legionellae have also been shown to survive in marine environments (42). Their natural hosts, protozoans like amoebae, can serve as transmission vehicles and protective shell against disinfection or heat treatment (3, 21, 22).

In their natural freshwater habitats, legionellae are rarely the causative agents of disease because the uptake mechanisms that are necessary for infection of humans are not given, e.g. no small droplets are produced. Isolation attempts of *L. pneumophila* from aquatic environments often fail due to different reasons. First of all, the fastidious microorganism grows relatively slow and plates are often overgrown by competing bacteria. On the other hand, especially in hot water systems, legionellae can loose their culturability and enter a viable but nonculturable (VBNC) state (see also Fig. 3). The VBNC state is the main reason why *L. pneumophila* cannot be isolated from natural and man-made aquatic environments which are suspected to be the source of infection (96).

Epidemiological analyses of infections caused by *L. pneumophila* depend on the accurate identification of strains, preferably at the clonal level. For the typing of *L. pneumophila* isolates, several methods have been applied in the last years, for example multiple-locus sequence typing (MLST) which is based on DNA sequencing of multiple polymorphic DNA segments (34, 84). The method was considered as more reproducible than the standard analysis for *L. pneumophila* SG 1 isolates via Amplified fragment length polymorphism (AFLP) analysis (33). In a simpler sequence-based typing (SBT) scheme, six genes of SG 1 isolates (namely *flaA, pilE, asd, mip, mompS*, and *proA*) were investigated for

the typing of clinical *L. pneumophila* isolates (34). In a study of an outbreak, which occurred in summer 2003 in Rome (Italy), SBT was reported to be the most rapid and easy to perform approach by comparison with two other typing methods (AFLP and pulse-field gel electrophoresis) (89). Very recently, a multiple-locus variable-number of tandem repeat (VNTR) analysis (MLVA) was applied and also approved by Eurosurveillance (82, 83). This method is used to determine the allele-related repeat size variation of eight different VNTR loci and was also adapted to capillary electrophoresis for a fast, reproducible and low-cost genotyping for *L. pneumophila* isolates (72). All these tools for epidemiological typing allow the assignment of an isolate to a previously defined type. Recently, web-based libraries (http://minisatellites.u-psud.fr/) have been implemented allowing comparison of isolates without the need of strain transfer between laboratories (83).

1.7 High resolution genotyping of bacterial pathogens

In general, typing methods are used to study the spread and population dynamics of a bacterial species in clinical and environmental settings. Genomic or phenotypic diversity can occur within a population of the same bacterial species. Isolates that are clonally related can be identified with the use of epidemiological markers. These markers are designed to discriminate on the subspecies level between related and unrelated isolates of the pathogen under investigation (68, 97). The usefulness of a marker is strongly dependent on its stability within a strain and its diversity within a species (68). Well-established “conventional” typing methods such as bacteriophage typing (108), serotyping (104, 110) or biochemical typing (75) have been contributed to the understanding of epidemiology of clinical relevant bacterial species like *Salmonella* spp. or *Escherichia coli*.

In the last years, high resolution molecular typing methods using genomic polymorphisms have become a powerful tool for the understanding of the epidemiology of infectious diseases (1, 68). Genotypic typing methods include a variety of different tools for the assessment of genomic variation in bacterial isolates. They can refer to composition (e.g. presence or absence of plasmids), overall structure (e.g. differing restriction endonuclease sites, number and positions of repetitive elements) or specific nucleotide sequences (e.g. single or several genes) of the genome. These typing systems require high reproducibility, consensus in interpretation and global communication platforms for proper assignment (40, 97). Other criteria for the usefulness of a typing method are also flexibility, speed, accessibility, ease of use and cost per analysis (6).

The first widely adopted application in this field, also used for molecular epidemiology of legionellae, was Restriction Endonuclease Analysis (REA) (100). REA belongs to the group of restriction fragment length polymorphisms (RFLP) which are based on the differing location of restriction sites within the genomes of isolates (79). Compared to other typing

methods, REA or RFLP turned out to be highly reproducible (15, 57, 109). Since reproducibility of a method strongly affects its discriminatory power it should be high for a reliable typing of strains (97). If “rare cutter” restriction enzymes are used for digestion, the resulting large fragments (up to 600kbp) can be separated by Pulsed Field Gel Electrophoresis (PFGE) which has a high discriminatory power and reproducibility. Therefore, PFGE has become a widely accepted tool for typing of almost all bacterial species including legionellae (25). The method is commonly considered as the gold standard in epidemiological studies of pathogens and the database entries are therefore numerous. For example more than 100,000 *E. coli* and *S. enterica* strains have been analyzed by PFGE (98). Like for all fingerprint techniques, special attention has to be turned on standardization efforts.

Another, PCR-based, typing tool is Amplified Fragment Length Polymorphism (AFLP) designed to selectively amplify subsets of genomic fragments generated with one or two restriction enzymes, usually a “rare” and a “frequent cutter”. AFLP turned out to be a fast, efficient, and reproducible method for typing strains of *Legionella pneumophila* isolated from humans and also the environment (9, 105).

A sequence-based typing (SBT) method used very frequently and with high reproducibility is Multiple-locus sequence typing (MLST). In a typical MLST assay, several coding genes (usually housekeeping genes) are sequenced completely or in parts to differentiate between isolates. The first application of this tool was a study in which 107 isolates of *Neisseria meningitides*, obtained from diseased and healthy carriers, have been typed using 11 housekeeping genes (67). The species presented a challenge for other typing methods due to frequent recombination events between lineages (67). But despite recombination, MLST was able to identify hyper-virulent clones of the species. Very rapidly, more advantages over molecular methods that rely on the comparison of fragment sizes (e.g. RFLP), became obvious: the method is unambiguous, also suitable for clinical material, and since it produces sequence data, information can be shared among different laboratories in the world without exchanging strains (47). MLST is now developed for more than 48 microbial taxa and publicly available databases (e.g. http://pubmlst.org/) facilitate the assignment to previously detected isolates. For many human pathogens like *Clostridium difficile* (39), *Stenotrophomonas maltophilia* (52), *Campylobacter jejuni* (26) or *Candida* species (18) MLST has been effectively applied to elucidate population structures and to unravel the distribution of clinical isolates. For the typing of *Legionella pneumophila*, a set of six genes (34) was recommended by the European working group for *Legionella* infections (EWGLI) (www.ewgli.org). The discriminatory power to separate serogroup 1 strains could be increased recently by adding one more gene to the assay (84).

SNP (Single Nucleotide Polymorphism) genotyping constitutes the detection of a nucleotide base that is present in a given strain at defined nucleotide positions known to be variable within the population of a species (6). To reveal polymorphisms useful for genotyping via SNPs, mutation discovery has to be conducted for example by shotgun sequencing, comparative genome sequencing (106) or also sequencing of defined genes. One major advantage of SNP analyses is that it can be also applied to highly clonal bacterial species like *Bacillus anthracis* (85) or *Mycobacterium tuberculosis* (45). In these "genetically monomorphic" species, sequencing of few genes (like for MLST) will not result in an identification of polymorphisms that could help to elucidate their genetic population structure (1). Genetically monomorphic pathogens pose a technical challenge to the researcher because it is difficult to deduce evolutionary history due to very low sequence diversity (1). Based on new sequencing methodologies (e.g. pyrosequencing), the number of SNPs available for genotyping is increasing, thus facilitating new approaches to analyze evolution and genotype of bacteria, especially human pathogens. Although the method is not labour intensive, the prerequisite for SNP typing is that several genome sequences are available giving the possibility to compare genomes to identify SNPs. Additionally, the method has to be handled with care since errors in data analysis can lead to misinterpretation of results.

Four genomes of *Legionella pneumophila* serogroup 1 have been fully sequenced until now (Philadelphia 1, Lens, Paris, Corby). This allowed the detection of markers for genotyping and the development of a new PCR-based genotyping method for *L. pneumophila* strains. The multilocus variable number of tandem repeat (VNTR) analysis (MLVA) became a frequently used and widely accepted method also by the European working group for *Legionella* infections (EWGLI). MLVA is based on the variability present in many regions of repetitive DNA sequences (41, 53, 83). Through Slipped Strand Mispairing (SSM), that occurs during DNA replication, shortening or lengthening of the repeat region is caused by deletion or insertion of repeat units (5, 60). MLVA and VNTR have been compared with other genotyping methods and gained a broad application in molecular epidemiological studies (41, 53).

Based on the genome sequence of *L. pneumophila* strain Philadelphia, 25 different VNTR loci were characterized in 2003 by Pourcel et al. (82) with respect to their variability and use for genotyping. They tested their approach using *L. pneumophila* isolates of serogroups 1 to 14 (82). First, one very polymorphic marker was used to genotype colonies on agar plates. Later on, when additional *L. pneumophila* serogroup 1 genomes were accessible (strains Lens and Paris) and analyzed, the same research group identified several VNTR markers that could be used to genotype *L. pneumophila* serogroup 1 isolates (83). The PCR and, initially, gel-electrophoresis based method included the analysis of 8 different markers and was later on simplified by detection of fluorescently labelled PCR amplicons via

capillary electrophoresis (72). So far, the method did not allow genotyping of *L. pneumophila* based on *in situ* samples. However, cultivation based methods have more constraints especially for the analysis of clinical material (e.g. slow growth of *Legionella* spp., interference with antibiotic treatment, no use of frozen material for retrospective molecular epidemiology studies). Therefore, there is an urgent need for a tool which can be directly applied to environmental and clinical samples, enabling the genotyping of this emerging pathogen without the prerequisite of cultivation.

1.8 Objectives of the thesis

As the first critical health issue, the assessment of live and dead bacterial taxa in drinking water was performed. To achieve this objective a combination of cellular and molecular analyses was used. At first, we analyzed the community structure and composition of the drinking water microflora. For the distinction between live and dead bacterial taxa, we applied live/dead staining (using a combination of two fluorescent dyes, SYTO9 and Propidium Iodide) and FACS sorting. By concentrating the drinking water bacteria through filtration, the staining procedure could be applied to a large number of cells. Using Fluorescence Activated Cell Sorting (FACS), the live and dead fractions of drinking water bacteria were separated from each other, the size of the both fractions were determined and live and dead fractions were analysed by fingerprinting and sequencing. By this approach, phylotypes in the live and dead fraction could be successfully assigned and abundances could be compared to abundances in the unsorted microflora.

As second critical health issue, the *in-situ* high resolution detection of a health relevant pathogenic bacterium was achieved. Species of the genus *Legionella* occur in most drinking water supply systems (DWSS) and pose a significant health threat in case the most infectious species and serotype, i.e. *Legionella pneumophila* serogroup 1, is present. Therefore, the second objective of this thesis was to determine which species of the genus Legionella are present in drinking water and, if *Legionella pneumophila* is present, to identify the genotype without cultivation. To achieve this objective a nested hierarchical approach was chosen. This approach comprised different steps. First, members of the genus *Legionella* present in drinking water samples were determined using a genus-specific PCR screening. Therefore, a genus-specific primer pair was developed, tested and applied to a set of drinking water extracts. In the next step, *L. pneumophila* was quantified in drinking water samples obtained from the HZI campus with specific real-time PCR. In addition, we obtained *L. pneumophila* isolates from our drinking water, the hot water circulation and the cooling towers on the campus. These isolates were used to test a new approach for high-resolution genotyping of *L. pneumophila* isolates in environmental samples, i.e. we adapted and further developed the approved MLVA-8 typing scheme for *L. pneumophila* to our SSCP

gel electrophoresis. For the first time, this approach enabled not only the detection of different genotypes in one sample but also the possibility for sequencing of the VNTR products from the gel and therefore an *in-situ* high resolution genotyping of different *L. pneumophila* strains in one environmental sample.

1.9 References

1. **Achtman M.** 2008. Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. Annu. Rev. Microbiol. **62**:53-70.

2. **Adeleke A. A., B. S. Fields, R. F. Benson, M. I. Daneshvar, J. M. Pruckler, R. M. Ratcliff, T. G. Harrison, R. S. Weyant, R. J. Birtles, D. Raoult, and M. A. Halablab**. 2001. *Legionella drozanskii* sp. nov., *Legionella rowbothamii* sp. nov. and *Legionella fallonii* sp. nov.: three unusual new *Legionella* species. Int. J. Syst. Evol. Microbiol **51**:1151-1160.

3. **Atlas R. M.** 1999. *Legionella*: from environmental habitats to disease pathology, detection and control. Environ. Microbiol **1**:283-293.

4. **Bartram J., Y. Chartier, J. V. Lee, and K. Pond**. 2007. *Legionella* and the prevention of legionellosis. WHO.

5. **van Belkum A., S. Scherer, L. van Alphen, and H. Verbrugh**. 1998. Short-sequence DNA repeats in prokaryotic genomes. Microbiol. Mol. Biol. Rev **62**:275-293.

6. **van Belkum A., P. T. Tassios, L. Dijkshoorn, S. Haeggman, B. Cookson, N. K. Fry, V. Fussing, J. Green, E. Feil, P. Gerner-Smidt, and others**. 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin Microbiol Infect **13 Suppl 3**:1-46.

7. **Benson C. A., and J. J. Ellner**. 1993. *Mycobacterium avium* Complex Infection and AIDS: Advances in Theory and Practice. Clin Infect Dis **17**:7-20.

8. **Benson R. F., and B. S. Fields**. 1998. Classification of the genus *Legionella*. Semin. Respir. Infect **13**:90-99.

9. **Bernander S., K. Jacobson, J. H. Helbig, P. C. Luck, and M. Landholm**. 2003. A Hospital-Associated Outbreak of Legionnaires' Disease Caused by *Legionella pneumophila* Serogroup 1 Is Characterized by Stable Genetic Fingerprinting but Variable Monoclonal Antibody Patterns. J. Clin. Microbiol. **41**:2503-2508.

10. **Berney M., F. Hammes, F. Bosshard, H. Weilenmann, and T. Egli**. 2007. Assessment and Interpretation of Bacterial Viability by Using the LIVE/DEAD BacLight Kit in Combination with Flow Cytometry. Appl. Environ. Microbiol. **73**:3283-3290.

11. **Berney M., H. U. Weilenmann, and T. Egli**. 2006. Flow-cytometric study of vital cellular functions in *Escherichia coli* during solar disinfection (SODIS). Microbiology **152**:1719-1729.

12. **Bhupathiraju V. K., M. Hernandez, D. Landfear, and L. Alvarez-Cohen**. 1999. Application of a tetrazolium dye as an indicator of viability in anaerobic bacteria. J Microbiol Methods **37**:231-243.

13. **Blázquez R. M., F. J. Espinosa, C. M. Martínez-Toldos, L. Alemany, M. C. García-Orenes, and M. Segovia**. 2005. Sensitivity of urinary antigen test in relation to clinical severity in a large outbreak of *Legionella pneumonia* in Spain. Eur J Clin Microbiol Infect Dis **24**:488-491.

14. **Boulos L., M. Prevost, B. Barbeau, J. Coallier, and R. Desjardins**. 1999. LIVE/DEAD BacLight : application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J Microbiol Methods **37**:77-86.

15. **Bowman R. A., G. L. O'Neill, and T. V. Riley**. 1991. Non-radioactive restriction fragment length polymorphism (RFLP) typing of *Clostridium difficile*. FEMS Microbiol Lett **79**:269-272.

16. **Breiman R. F., and J. C. Butler**. 1998. Legionnaires' disease: clinical, epidemiological, and public health perspectives. Semin Respir Infect **13**:84-89.

17. **Brettar I., and M. G. Höfle**. 2008. Molecular assessment of bacterial pathogens - a contribution to drinking water safety. Curr Opin Biotechnol **19**:274-280.

18. **Brillowska-Dabrowska A., O. Bergmann, I. M. Jensen, J. Jarløv, and M. C. Arendrup**. 2010. Typing of Candida isolates from patients with invasive infection and concomitant colonization. Scand J Infect Dis **42**:109-113.

19. **Chilton P., N. S. Isaacs, P. Manas, and B. M. Mackey**. 2001. Biosynthetic requirements for the repair of membrane damage in pressure-treated *Escherichia coli*. Int J Food Microbiol **71**:101–104.

20. **Clarridge J. E.** 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev **17**:840-862.

21. **Colbourne J. S., and P. J. Dennis**. 1985. Distribution and persistence of *Legionella* in water systems. Microbiol. Sci **2**:40-43.

22. **Colbourne J. S., P. J. Dennis, R. M. Trew, C. Berry, and G. Vesey**. 1988. *Legionella* and public water supplies. Water Sci Technol **20**:5-10.

23. **Colwell R. R.** 2000. Viable but nonculturable bacteria: a survival strategy. J Infect Chemother **6**:121-125.

24. **Czechowska K., D. R. Johnson, and J. R. van der Meer**. 2008. Use of flow cytometric methods for single-cell analysis in environmental microbiology. Curr. Opin. Microbiol **11**:205-212.

25. **De Zoysa A. S., and T. G. Harrison**. 1999. Molecular typing of *Legionella pneumophila* serogroup 1 by pulsed-field gel electrophoresis with Sfil and comparison of this method with restriction fragment-length polymorphism analysis. J Med Microbiol **48**:269-278.

26. **Dingle K. E., F. M. Colles, D. R. A. Wareing, R. Ure, A. J. Fox, F. E. Bolton, H. J. Bootsma, R. J. L. Willems, R. Urwin, and M. C. J. Maiden**. 2001. Multilocus Sequence Typing System for *Campylobacter jejuni*. J. Clin. Microbiol. **39**:14-23.

27. **Eichler S., M. G. Weinbauer, D. Dominik, and M. Höfle**. 2004. Extraction of total RNA and DNA from bacterioplankton, chapter 1.0.8, S. 103-120. *In* G.A.Kowalchuk; F.J.D.Bruijn; I.M.Head; A.D.L.Akkermans; and J.D.van Elsas (Ed.), Molecular microbial ecology manual, 2. Edition. Kluwer Academic Publishers, Dordrecht, The Netherlands.

28. **Eichler S., R. Christen, C. Höltje, P. Westphal, J. Bötel, I. Brettar, A. Mehling, and M. G. Höfle**. 2006. Composition and Dynamics of Bacterial Communities of a Drinking Water Supply System as Assessed by RNA- and DNA-Based 16S rRNA Gene Fingerprinting. Appl. Environ. Microbiol. **72**:1858–1872.

29. **Fields B. S.** 1996. The molecular ecology of legionellae. Trends Microbiol **4**:286-290.

30. **Fields B. S.** 1997. Legionellae and Legionnaires' disease. Manual of environmental microbiology **1**:666–675.

31. **Ford T. E.** 1999. Microbiological safety of drinking water: United States and global perspectives. Environ. Health Perspect **107 Suppl 1**:191-206.

32. **Fraser D. W., T. R. Tsai, W. Orenstein, W. E. Parkin, H. J. Beecham, R. G. Sharrar, J. Harris, G. F. Mallison, S. M. Martin, J. E. McDade, C. C. Shepard, and P. S. Brachman**. 1977. Legionnaires' disease: description of an epidemic of pneumonia. N. Engl. J. Med **297**:1189-1197.

33. **Fry N. K., B. Afshar, P. Visca, D. Jonas, J. Duncan, E. Nebuloso, A. Anderwood, and T. G. Harrison**. 2005. Assessment of fluorescent amplified fragment length polymorphism analysis for epidemiological genotyping of *Legionella pneumophila* serogroup 1. Clin. Microbiol. Rev. **11**:704-712.

34. **Gaia V., N. K. Fry, B. Afshar, P. C. Luck, H. Meugnier, J. Etienne, R. Peduzzi, and T. G. Harrison**. 2005. Consensus sequence-based scheme for epidemiological typing of clinical and environmental isolates of *Legionella pneumophila*. J Clin Microbiol **43**:2047-2052.

35. **Gasol J., U. Zweifel, F. Peters, J. Fuhrman, and A. Hagstrom**. 1999. Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. Appl.Environ.Microbiol **65**:4475-4483.

36. **Giovannoni S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field**. 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature **345**:60-63.

37. **Glick T. H., M. B. Gregg, B. Berman, G. Mallison, W. W. Rhodes, and I. Kassanoff**. 1978. Pontiac fever. An epidemic of unknown etiology in a health department: I. Clinical and epidemiologic aspects. Am. J. Epidemiol **107**:149-160.

38. **Glockner F. O., B. M. Fuchs, and R. Amann**. 1999. Bacterioplankton Compositions of Lakes and Oceans: a First Comparison Based on Fluorescence In Situ Hybridization. Appl. Environ. Microbiol. **65**:3721-3726.

39. **Griffiths D., W. Fawley, M. Kachrimanidou, R. Bowden, D. W. Crook, R. Fung, T. Golubchik, R. M. Harding, K. J. M. Jeffery, K. A. Jolley, R. Kirton, T. E. Peto, G. Rees, N. Stoesser, A. Vaughan, A. S. Walker, B. C. Young, M. Wilcox, and K. E. Dingle**. 2010. Multilocus Sequence Typing of *Clostridium difficile*. J. Clin. Microbiol. **48**:770-778.

40. **Grissa I., P. Bouchon, C. Pourcel, and G. Vergnaud**. 2008. On-line resources for bacterial micro-evolution studies using MLVA or CRISPR typing. Biochimie **90**:660-668.

41. **Harth-Chu E., R. T. Espejo, R. Christen, C. A. Guzman, and M. G. Höfle**. 2009. Multiple-Locus Variable-Number Tandem-Repeat Analysis for Clonal Identification of *Vibrio parahaemolyticus* Isolates by Using Capillary Electrophoresis. Appl. Environ. Microbiol. **75**:4079-4088.

42. **Heller R., C. Höller, R. Süssmuth, and K. O. Gandermann**. 1998. Effect of salt concentration and temperature on survival of *Legionella pneumophila*. Lett Appl Microbiol **26**:64–68.

43. **Henne K., L. Kahlisch, J. Draheim, I. Brettar, and M. G. Höfle**. 2008. Polyvalent fingerprint based molecular surveillance methods for drinking water supply systems. Water Science & Technology: Water Supply **8**:527-532.

44. **Hoefel D., P. Monis, W. Grooby, S. Andrews, and C. Saint**. 2005. Profiling bacterial survival through a water treatment process and subsequent distribution system. J Appl Microbiol **99**:175-186.

45. **Hughes A. L., R. Friedman, and M. Murray**. 2002. Genomewide Pattern of Synonymous Nucleotide Substitution in Two Complete Genomes of *Mycobacterium tuberculosis*. Emerg Infect Dis **8**:1342-1346.

46. **Huq A., I. N. Rivera, and R. R. Colwell**. 2000. Epidemiological significance of viable but nonculturable microorganisms, p. 301–323. *In* Nonculturable Microorganisms in the Environment.

47. **Jolley K. A.** 2009. Internet-based sequence-typing databases for bacterial molecular epidemiology. Methods Mol Biol **551**:305-312.

48. **Jones J. G.** 1977. The effect of environmental factors on estimated viable and total populations of planktonic bacteria in lakes and experimental enclosures. Freshwater Biol **7**:67–91.

49. **Joseph C. A., and European Working Group for Legionella Infections**. 2004. Legionnaires' Disease in Europe 2000–2002. Epidemiol. Infect. **132**:417-424.

50. **Josephson K. L., C. P. Gerba, and I. L. Pepper**. 1993. Polymerase chain reaction detection of nonviable bacterial pathogens. Appl. Environ. Microbiol. **59**:3513-3515.

51. **Joux F., and P. Lebaron**. 2000. Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. Microbes.Infect. **2**:1523-1535.

52. **Kaiser S., K. Biehler, and D. Jonas**. 2009. A *Stenotrophomonas maltophilia* Multilocus Sequence Typing Scheme for Inferring Population Structure. J Bacteriol **191**:2934-2943.

53. **Keim P., L. B. Price, A. M. Klevytska, K. L. Smith, J. M. Schupp, R. Okinaka, P. J. Jackson, and M. E. Hugh-Jones**. 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J. Bacteriol. **182**:2928-2936.

54. **Klein P., and V. Juneja**. 1997. Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. Appl. Environ. Microbiol. **63**:4441-4448.

55. **van der Kooij D.** 1992. Assimilable organic carbon as an indicator of bacterial regrowth. AWWA **84**:57–65.

56. **van der Kooij D., W. A. M. Hijnen, and J. C. Kruithof**. 1989. The Effects of Ozonation, Biological Filtration and Distribution on the Concentration of Easily Assimilable Organic Carbon (AOC) in Drinking Water. Ozone: Science & Engineering **11**:297-311.

57. **Kremer K., D. van Soolingen, R. Frothingham, W. H. Haas, P. W. M. Hermans, C. Martin, P. Palittapongarnpim, B. B. Plikaytis, L. W. Riley, M. A. Yakrus, J. M. Musser, and J. D. A. van Embden**. 1999. Comparison of Methods Based on Different Molecular Epidemiological Markers for Typing of *Mycobacterium tuberculosis* Complex Strains: Interlaboratory Study of Discriminatory Power and Reproducibility. J. Clin. Microbiol. **37**:2607-2618.

58. **LeChevallier M. W.** 1990. Coliform regrowth in drinking water: a review. AWWA **82**:74–86.

59. **Lew J. F., R. I. Glass, R. E. Gangarosa, I. P. Cohen, C. Bern, and C. L. Moe**. 1991. Diarrheal deaths in the United States, 1979 through 1987. A special problem for the elderly. JAMA **265**:3280-3284.

60. **Lindstedt B.** 2005. Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. Electrophoresis **26**:2567-2582.

61. **Lindström E.** 2001. Investigating Influential Factors on Bacterioplankton Community Composition: Results from a Field Study of Five Mesotrophic Lakes. Microb. Ecol **42**:598-605.

62. **Lindström E. S., M. P. Kamst-Van Agterveld, and G. Zwart**. 2005. Distribution of typical freshwater bacterial groups is associated with pH, temperature, and lake water retention time. Appl. Environ. Microbiol **71**:8201-8206.

63. **Liu W., H. Wu, Z. Wang, S. Ong, J. Hu, and W. Ng**. 2002. Investigation of assimilable organic carbon (AOC) and bacterial regrowth in drinking water distribution system. Water Res **36**:891-898.

64. **Llobet-Brossa E., R. Rossello-Mora, and R. Amann**. 1998. Microbial Community Composition of Wadden Sea Sediments as Revealed by Fluorescence In Situ Hybridization. Appl. Environ. Microbiol. **64**:2691-2696.

65. **Lo Presti F., S. Riffard, H. Meugnier, M. Reyrolle, Y. Lasne, P. A. Grimont, F. Grimont, R. F. Benson, D. J. Brenner, A. G. Steigerwalt, J. Etienne, and J. Freney**. 2001. *Legionella gresilensis* sp. nov. and *Legionella beliardensis* sp. nov., isolated from water in France. Int. J. Syst. Evol. Microbiol **51**:1949-1957.

66. **Lo Presti F., S. Riffard, H. Meugnier, M. Reyrolle, Y. Lasne, P. A. Grimont, F. Grimont, F. Vandenesch, J. Etienne, J. Fleurette, and J. Freney**. 1999. *Legionella taurinensis* sp. nov., a new species antigenically similar to Legionella spiritensis. Int. J. Syst. Bacteriol **49 Pt 2**:397-403.

67. **Maiden M. C., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, and others**. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A **95**:3140-3145.

68. **Maslow J. N., M. E. Mulligan, and R. D. Arbeit**. 1993. Molecular Epidemiology: Application of Contemporary Techniques to the Typing of Microorganisms. Clin Infect Dis **17**:153-162.

69. **Morris R. M., M. S. Rappe, S. A. Connon, K. L. Vergin, W. A. Siebold, C. A. Carlson, and S. J. Giovannoni**. 2002. SAR11 clade dominates ocean surface bacterioplankton communities. Nature **420**:806-810.

70. **Murdoch D. R.** 2003. Diagnosis of *Legionella* infection. Clin Infect Dis **36**:64–69.

71. **Nebe-von-Caron G., Stephens, and Badley**. 1998. Assessment of bacterial viability status by flow cytometry and single cell sorting. J Appl Microbiol **84**:988-998.

72. **Nederbragt A. J., A. Balasingham, R. Sirevåg, H. Utkilen, K. S. Jakobsen, and M. J. Anderson-Glenna**. 2008. Multiple-locus variable-number tandem repeat analysis of *Legionella pneumophila* using multi-colored capillary electrophoresis. J. Microbiol. Methods **73**:111-117.

73. **Nocker A., and A. K. Camper**. 2006. Selective Removal of DNA from Dead Cells of Mixed Bacterial Communities by Use of Ethidium Monoazide. Appl. Environ. Microbiol. **72**:1997-2004.

74. **Nocker A., C. Cheung, and A. K. Camper**. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods **67**:310-320.

75. **O'Hara C. M., F. W. Brenner, and J. M. Miller**. 2000. Classification, Identification, and Clinical Significance of *Proteus*, *Providencia*, and *Morganella*. Clin. Microbiol. Rev. **13**:534-546.

76. **Oliver J. D.** 2000. The public health significance of viable but nonculturable bacteria, S. 277–300. *In* Nonculturable microorganisms in the environment. ASM Press, Washington, DC.

77. **Oliver J.** 2005. The viable but nonculturable state in bacteria. J Microbiol **43 Spec No**:93-100.

78. **Olsen G. J., D. J. Lane, S. J. Giovannoni, N. R. Pace, and D. A. Stahl**. 1986. Microbial Ecology and Evolution: A Ribosomal RNA Approach. Annu. Rev. Microbiol. **40**:337-365.

79. **Owen R. J.** 1989. Chromosomal DNA fingerprinting–a new method of species and strain identification applicable to microbial pathogens. J Med Microbiol **30**:89-99.

80. **Petti C. A., C. R. Polage, and P. Schreckenberger**. 2005. The Role of 16S rRNA Gene Sequencing in Identification of Microorganisms Misidentified by Conventional Methods. J Clin Microbiol **43**:6123-6125.

81. **Poglazova M.** 1996. A spectrofluorimetric method for the determination of total bacterial counts in environmental samples. J Microbiol Methods **24**:211-218.

82. **Pourcel C., Y. Vidgop, F. Ramisse, G. Vergnaud, and C. Tram**. 2003. Characterization of a Tandem Repeat Polymorphism in *Legionella pneumophila* and Its Use for Genotyping. J. Clin. Microbiol. **41**:1819-1826.

83. **Pourcel C., P. Visca, B. Afshar, S. D'Arezzo, G. Vergnaud, and N. K. Fry**. 2007. Identification of Variable-Number Tandem-Repeat (VNTR) Sequences in *Legionella pneumophila* and Development of an Optimized Multiple-Locus VNTR Analysis Typing Scheme. J. Clin. Microbiol. **45**:1190-1199.

84. **Ratzow S., V. Gaia, J. H. Helbig, N. K. Fry, and P. C. Luck**. 2007. Addition of neuA, the Gene Encoding N-Acylneuraminate Cytidylyl Transferase, Increases the Discriminatory Ability of the Consensus Sequence-Based Scheme for Typing *Legionella pneumophila* Serogroup 1 Strains. J. Clin. Microbiol. **45**:1965-1968.

85. **Read T. D., S. L. Salzberg, M. Pop, M. Shumway, L. Umayam, L. Jiang, E. Holtzapple, J. D. Busch, K. L. Smith, J. M. Schupp, D. Solomon, P. Keim, and C. M. Fraser**. 2002. Comparative Genome Sequencing for Discovery of Novel Polymorphisms in *Bacillus anthracis*. Science **296**:2028-2033.

86. **Roszak D. B., and R. R. Colwell**. 1987. Survival strategies of bacteria in the natural environment. Microbiol Mol Biol Rev **51**:365-379.

87. **Rudi K., B. Moen, S. M. Dromtorp, and A. L. Holck**. 2005. Use of Ethidium Monoazide and PCR in Combination for Quantification of Viable and Dead Cells in Complex Samples. Appl. Environ. Microbiol. **71**:1018-1024.

88. **Saiki R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mulls, and H. A. Erlich**. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.

89. **Scaturro M., M. Losardo, G. De Ponte, and M. L. Ricci**. 2005. Comparison of Three Molecular Methods Used for Subtyping of *Legionella pneumophila* Strains Isolated during an Epidemic of Legionellosis in Rome. J Clin Microbiol **43**:5348-5350.

90. **Schmalenberger A., F. Schwieger, and C. C. Tebbe**. 2001. Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. Appl. Environ. Microbiol **67**:3557-3563.

91. **Schmidt T. M., E. F. DeLong, and N. R. Pace**. 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. **173**:4371-4378.

92. **Schwartz T., S. Hoffmann, and U. Obst**. 1998. Formation and bacterial composition of young, natural biofilms obtained from public bank-filtered drinking water systems. Water Res **32**:2787-2797.

93. **Smith J. J., and G. A. McFeters**. 1997. Mechanisms of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride), and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) reduction in Escherichia coli K-12. J Microbiol Methods **29**:161-175.

94. **Stahl D. A., D. J. Lane, G. J. Olsen, and N. R. Pace**. 1984. Analysis of Hydrothermal Vent-Associated Symbionts by Ribosomal RNA Sequences. Science **224**:409-411.

95. **Staley J. T., and A. Konopka**. 1985. Measurement of in Situ Activities of Nonphotosynthetic Microorganisms in Aquatic and Terrestrial Habitats. Annu. Rev. Microbiol. **39**:321-346.

96. **Steinert M., U. Hentschel, and J. Hacker**. 2002. *Legionella pneumophila*: an aquatic microbe goes astray. FEMS Microbiol Rev **26**:149-162.

97. **Struelens M. J., A. Bauernfeind, A. Van Belkum, D. Blanc, B. D. Cookson, L. Dijkshoorn, N. El Solh, J. Etienne, J. Garaizar, P. Gerner-Smidt, and others**. 1996. Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. Clin Microbiol Infect **2**:2-11.

98. **Swaminathan B., T. J. Barrett, S. B. Hunter, R. V. Tauxe, and P. N. CDC**. 2001. PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg Infect Dis **7**:382–389.

99. **Templeton K. E., S. A. Scheltinga, P. Sillekens, J. W. Crielaard, A. P. van Dam, H. Goossens, and E. C. J. Claas**. 2003. Development and Clinical Evaluation of an Internally Controlled, Single-Tube Multiplex Real-Time PCR Assay for Detection of *Legionella pneumophila* and Other *Legionella* Species. J. Clin. Microbiol. **41**:4016-4021.

100. **Tompkins L. S., N. J. Troup, T. Woods, W. Bibb, and R. M. McKinney**. 1987. Molecular epidemiology of *Legionella* species by restriction endonuclease and alloenzyme analysis. J. Clin. Microbiol. **25**:1875-1880.

101. **Torsvik V., J. Goksoyr, and F. L. Daae**. 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. **56**:782-787.

102. **Tsai Y. P., T. Y. Pai, and J. M. Qiu**. 2004. The impacts of the AOC concentration on biofilm formation ander higher shear force condition. J Biotechnol **111**:155–167.

103. **Ullrich S., B. Karrasch, H. Hoppe, K. Jeskulke, and M. Mehrens**. 1996. Toxic Effects on Bacterial Metabolism of the Redox Dye 5-Cyano-2,3-Ditolyl Tetrazolium Chloride. Appl. Environ. Microbiol. **62**:4587-4593.

104. **Uzzau S., D. J. Brown, T. Wallis, S. Rubino, G. Leori, S. Bernard, J. Casadesus, D. J. Platt, and J. E. Olsen**. 2001. Host adapted serotypes of *Salmonella enterica*. Epidemiol Infect **125**:229–255.

105. **Valsangiacomo C., F. Baggi, V. Gaia, T. Balmelli, R. Peduzzi, and J. Piffaretti**. 1995. Use of amplified fragment length polymorphism in molecular typing of *Legionella pneumophila* and application to epidemiological studies. J. Clin. Microbiol. **33**:1716-1719.

106. **Van Ert M. N., W. R. Easterday, T. S. Simonson, J. M. U'Ren, T. Pearson, L. J. Kenefic, J. D. Busch, L. Y. Huynh, M. Dukerich, C. B. Trim, J. Beaudry, A. Welty-Bernard, T. Read, C. M. Fraser, J. Ravel, and P. Keim**. 2007. Strain-Specific Single-Nucleotide Polymorphism Assays for the *Bacillus anthracis* Ames Strain. J. Clin. Microbiol. **45**:47-53.

107. **Weber J. T., W. C. Levine, D. P. Hopkins, and R. V. Tauxe**. 1994. Cholera in the United States, 1965-1991: risks at home and abroad. Arch Intern Med **154**:551-556.

108. **Wentworth B. B.** 1963. Bacteriophage typing of the staphylococci. Microbiol Mol Biol Rev **27**:253-272.

109. **Wichelhaus T. A., K. P. Hunfeld, B. B\öddinghaus, P. Kraiczy, V. Sch\äfer, and V. Brade**. 2001. Rapid molecular typing of methicillin resistant *Staphylococcus aureus* by PCR-RFLP. Infect Control Hosp Epidemiol **22**:294–298.

110. **Wolf M.** 1997. Occurrence, distribution, and associations of O and H serogroups, colonization factor antigens, and toxins of enterotoxigenic *Escherichia coli*. Clin. Microbiol. Rev. **10**:569-584.

111. **Xu H., N. Roberts, F. L. Singleton, R. W. Attwell, D. J. Grimes, and R. R. Colwell**. 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb Ecol **8**:313-323.

112. **Yu V. L., J. F. Plouffe, M. C. Pastoris, J. E. Stout, M. Schousboe, A. Widmer, J. Summersgill, T. File, C. M. Heath, D. L. Paterson, and A. Chereshsky**. 2002. Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative survey. J. Infect. Dis. **186**:127-128.

113. **Zwart G., B. C. Crump, M. P. Kamst-van Agterveld, F. Hagen, and S. K. Han**. 2002. Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquat Microb Ecol **28**:141–155.

CHAPTER 2

Molecular analysis of the bacterial drinking water community with respect to live/dead status

Leila Kahlisch[1], Karsten Henne[1], Lothar Gröbe[2], Josefin Draheim[1], Manfred G. Höfle[1] and Ingrid Brettar[1]

[1]Dept. Vaccinology and Applied Microbiology, Helmholtz Center for Infection Research (HZI), Inhoffenstrasse 7, 38124 Braunschweig, Germany

[2] Department of Cell Biology, Helmholtz Center for Infection Research (HZI), Inhoffenstrasse 7, 38124 Braunschweig, Germany

CHAPTER 2 Molecular analysis of the bacterial drinking water community with respect to live/dead status

2.1 Abstract

The assessment of the physiological state of the bacteria in drinking water is a critical issue, especially with respect to the presence of pathogenic bacteria. Though molecular methods can provide insight into the taxonomic composition of the drinking water microflora, the question if a bacterial species is alive or dead still needs to be addressed. To distinguish live and dead bacteria at the taxonomic level, we combined three methods; i) a staining procedure indicating membrane-injured cells (using SYTO9 and Propidium Iodide) that is considered to distinguish between live and dead cells, ii) a Fluorescence Activated Cell Sorting (FACS) of the membrane injured and intact bacteria, and iii) molecular analyses of the RNA extracted from the bacteria before and after sorting to analyze the bacterial community at the species level. By staining and FACS analysis the drinking water bacteria could be separated according to their different membrane integrities, and RNA could be extracted from the live and dead sorted bacterial fractions. 16S rRNA based fingerprints revealed a diverse bacterial community in the drinking water samples with the majority being represented by 31 identified phylotypes. Most of the phylotypes belonged to the phyla Proteobacteria (Alpha-, Beta-, Gamma-), Cyanobacteria and Bacteroidetes, and were mostly related to freshwater bacteria. 90% of the total phylotypes could be recovered after FACS-Sorting; 32% of the phylotypes occurred only in the “live” sorted fraction, 21% only in the “dead” sorted fraction, and 46% occurred in both fractions.

2.2 Introduction

Despite the water treatment process aims at eliminating or killing the bacteria, drinking water still shows a diverse microflora, partially due to re-growth after the treatment process (5). Molecular methods such as 16S rRNA based fingerprints and sequencing can provide insight into the taxonomic composition of the drinking water microflora (8). However, the physiological state of the bacteria is a critical question and still remains to be assessed. On the one hand, molecular methods are not affected by the problem due to viable but non-culturable bacteria (VNBC) hampering cultivation based methods, i.e. that VNBC-bacteria are not growing on the respective media despite the fact that they are still alive and infective (11). On the other hand, it was often observed that bacteria, including pathogenic ones, were detected by molecular methods, but the viability of the cells could not be confirmed (14). It is of great health relevance for drinking water, especially with respect to pathogenic bacteria, to distinguish live and dead bacteria at the taxonomic level, i.e. to estimate the live and dead fraction of each bacterial species in drinking water.

For assessing the viability of bacterial cells at a single-cell level without the use of cultivation methods, several fluorescent dyes are available that can be applied in epifluorescence microscopy or flow cytometry (1, 3). These dyes turned out to be a valuable tool for the understanding of viability and cell integrity. The combination of two nucleic acid stains Propidium Iodide (PI) and Syto9 is often used to distinguish live and dead bacterial cells. The green fluorescent stain Syto9 is able to pass intact bacterial membranes, and is used as marker for all bacterial cells. Propidium iodide (PI) is a red fluorescent dye which is only able to pass the cytoplasmic membrane in case of a damaged membrane (2, 4).

The HEALTHY-WATER project, a project in the 6th Framework of the EU (http://www.hzi-helmholtz.de/en/healthy_water/) is aiming towards the development of new molecular detection technologies of microbial pathogens in drinking water with special emphasis on emerging pathogens (15). Among several approaches that are under development, fingerprint based methods are used because they have the potential to monitor the whole bacterial community and thus bear the potential to detect also unexpected pathogenic bacteria. RNA based fingerprints were used because they are considered to represent the active part of the bacterial drinking water community (8), an assumption based on the general observation that the number of ribosomes per bacterial cell is a good measure of its overall activity.

In this study, we applied a combination of staining, sorting by flow cytometry, fingerprinting and fingerprint sequencing to assess the viability of bacterial species present in samples of drinking water of the city of Braunschweig in Northern Germany. The studied drinking water was considered as an example for chlorinated drinking water derived from surface water, i.e. oligotrophic and dystrophic reservoirs.

2.3 Methods

2.3.1 Study site, sampling and cell counting

Drinking water samples were obtained on 3 days, i.e. 25 March 2008 (= sampling A), 31 March 2008 (= sampling B) and 5 May 2008 (= sampling C) from the tap in lab D0.04 of the Helmholtz-Centre for Infection Research, Braunschweig-Stöckheim, Germany. Sampling A and B were taken as samples where a high similarity was expected due to the short time interval, sampling C was considered to display a distinct community due to the previously observed seasonal changes (9). The drinking water originated from two surface water reservoirs (oligotrophic, and dystrophic water) situated in a mountain range 40 km south of Braunschweig. Water processing included flocculation/coagulation, sand filtration and chlorination (0.2 - 0.7 mg l-1). At the tap, the chlorine concentration was below the detection limit of 0.02 mg/l. More details on the respective drinking water supply system are given elsewhere (8).

Per sample, 18 litre of drinking water were filtered onto 0.2 µm pore size polycarbonate filters (90 mm diameter; Nuclepore; Whatman, Maidstone, United Kingdom), and used immediately for further processing: the biomass was scraped off the filter, a part of it was either immediately used for the staining procedure as indicated below, and a fraction of the biomass was immediately frozen for later molecular analysis (-70℃).

Total bacteria counts were obtained after staining with Sybr Green I dye (1:10000 final dilution; Molecular Probes, Invitrogen) using formaldehyde-fixed samples (2% final concentration). Heterotrophic plate counts were done on R2A agar medium (Oxoid) by incubating at 22℃ in the dark for 72 hours.

2.3.2 Molecular methodology

For distinction between membrane injured and intact bacteria, a part of the freshly harvested biomass was resuspended yielding a 100 to 400 concentrated solution of the drinking water bacteria. The concentrated bacteria were immediately stained with SYTO 9 and propidium iodide (PI) (BacLight Kit, Molecular Probes, Invitrogen) at final concentrations of 5 µM and 30 µM, respectively, according to the instructions of the manufacturer. After incubation for 20 min in the dark, FACS analysis was carried out using a MOFLO (Beckman Coulter, Krefeld, Germany) with a 488 nm laser (2). The bandpass filters used were 530/40 nm and 616/26 nm for SYTO 9 and PI, respectively. The sorted cells were harvested by centrifugation for 15 min at 15.000 xg and stored frozen (-70℃) for later nucleic acid extraction. Pellet supernatant was checked by epifluorescence microscopy for microorganisms; in no case cells were observed. For the workflow of the overall procedure see Fig. 1.

For molecular analysis of the bacteria by 16S rRNA based fingerprints, the RNA was extracted from the frozen biomass as described in more detail by Eichler at al. (8). In brief: RNA was extracted and purified; bacterial 16S rRNA gene amplicons generated by RT-PCR were subjected to separation by non-denaturing acrylamide gel electrophoresis enabling Single Strand Conformational Polymorphism (SSCP) analysis (16). RNA based SSCP analyses were performed to analyze the bacterial community before and after viability staining and sorting. The banding patterns on the SSCP gels, used as a direct measure of the community structure, were compared by cluster analysis (GelCompare II, Applied Maths). The composition of the bacterial community was determined by sequencing the single bands of the gel pattern following the protocol of Eichler et al. (8) and identifying the sequences by phylogenetic analysis using the international 16S rRNA gene sequence data base.

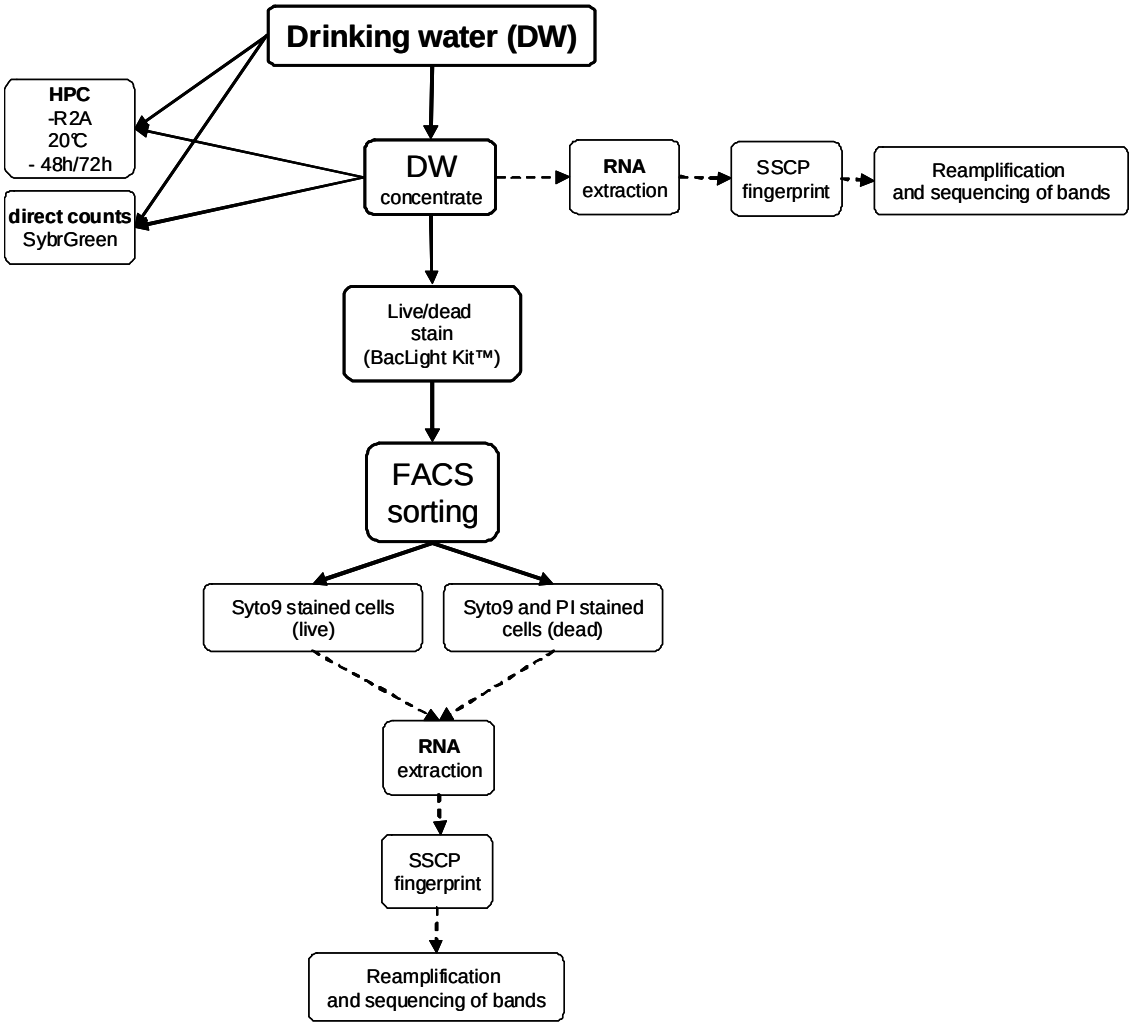


Fig.1. Flow chart of the analysis of drinking water samples using live/dead staining. Drinking water bacteria were concentrated 100-400 fold by filtration onto a 0.2 µm Nucleopore filter and scraped off into a 0.9 % NaCl solution. The microorganisms were stained with the BacLight Kit for 15-20 min in the dark. By FACS the differently stained cells of the drinking water bacteria were sorted according to their membrane integrity. From the FACS-sorted cells and the unsorted drinking water bacteria, RNA was extracted and subjected to single strand conformation polymorphism (SSCP) analysis (for details see Material & Methods).

2.4 Results and Discussion

2.4.1 Bacterial counts before and after sorting

For drinking water samples obtained from the tap at the three sampling dates, the total bacterial cell numbers were in the range of 3 to 4 x 105 cells ml-1; in the 100 to 400 fold concentrates of the drinking water bacteria used for viability staining the cell numbers ranged from 5.1 x 107 to 1.2 x 108 cells ml-1. Heterotrophic plate counts made from the concentrates on R2A agar ranged from 2 to 4.1 x 103 colony forming units (CFU) ml-1 and were thus substantially less than the total bacterial counts, i.e. by four to five orders of magnitude.

After staining by PI/SYTO9, the fraction of membrane intact cells counted microscopically accounted for 53% ± 6% of the total bacteria while the membrane injured fraction accounted for 47% ± 6%.

2.4.2 Analysis of drinking water bacteria by fingerprints before and after viability staining and sorting

With help of the FACS-sorting, the membrane injured red fluorescent cells were separated from the green fluorescent cells with a presumably intact cell membrane. After sorting, RNA was extracted from both of these fractions, that will be termed in the following as the “live” and the “dead” sorted fractions. SSCP fingerprints based on the extracted RNA for the sorted fractions and a part of the drinking water concentrate (not submitted to staining and sorting) are given in Figure 2.

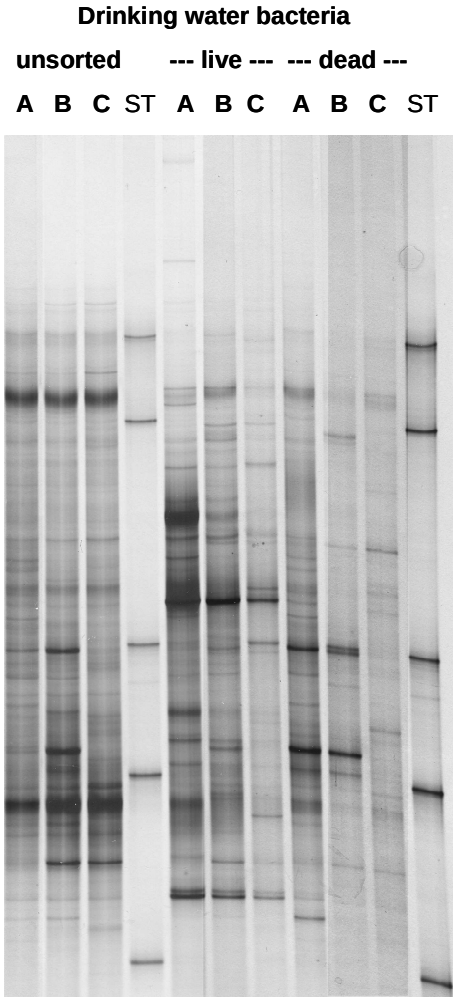


Fig. 2. 16S rRNA based SSCP fingerprints before and after Syto9/PI staining and FACS analysis of the drinking water bacteria. The figure shows the SSCP fingerprinting pattern for the three sampling dates (indicated by A (25.03.2008), B (31.03.2008) and C (05.05.2008) for the bacteria in the untreated, i.e. unstained and unsorted, drinking water concentrate (termed “unsorted”), and for the live (“live”) and dead sorted (“dead”) fraction. “ST” indicates the “SSCP gel standard” (composed out of amplicons of five indicative bacterial species).

The fingerprints of the drinking water bacteria showed a high similarity for the three sampling days. After sorting, the live sorted fraction showed a high similarity. This was different for the dead sorted fraction where especially the last sampling in May 2009 showed a very different pattern. The cluster analysis of the fingerprint of Figure 2 (Fig. 3) shows a similarity range from almost 90% to 40% for the lower similarity of the dead sorted fraction.

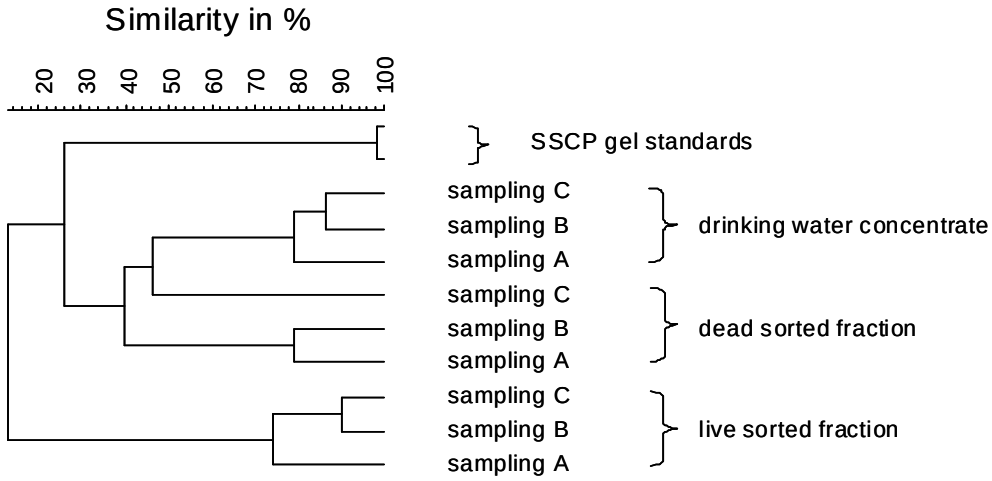


Fig. 3. Cluster analysis of the 16S rRNA based SSCP fingerprint given in figure 2 comparing the total bacterial community with the live and dead sorted fraction for the three different sampling dates. Similarity coefficients were calculated using Pearson correlation algorithm. Dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic mean. Sample designations are as in Fig. 2. The "SSCP gel standard" (composed out of amplicons of five bacterial species) was taken as out-group for the cluster analysis.

SSCP fingerprint analyses of the live and dead sorted fractions in comparison to the unsorted total bacterial community revealed that many bacterial species had live and dead cells, while some species could be found on either side, i.e. completely membrane injured or not injured at all (Fig. 2). This can be seen from the fingerprint patterns, and was confirmed by the sequencing of the major bands of the fingerprint and the respective phylogenetic analysis.

The drinking water microflora detected by the sequencing of the RNA based SSCP fingerprints was rather diverse and showed some variability per species among the different sampling dates. For the bacterial drinking water community a total of 31 phylotypes (based on 98% 16S rRNA sequence similarity) was detected. For the analyzed data set, most phylotypes can be regarded as specific species. The community was dominated by Proteobacteria (Alpha-, Beta-, Gammaproteobacteria), Cyanobacteria and Bacteroidetes. In addition, members of the Chloroflexi, Nitrospira, Firmicutes and Planctomycetes were observed. For the (unsorted) drinking water samples, the Betaproteobacteria formed the

largest fraction with an average of 21% of the total community, followed by the Cyanobacteria (16%), the Alphaproteobacteria (15%), the Gammaproteobacteria (10%), and the Bacteroidetes (8%). All other phyla contributed on average to less than 5%. From the 31 phylotypes, seven phylotypes were able to contribute to more than 5% (up to 24%) of the total drinking water community (in terms of band intensity per lane). These seven dominating phylotypes were composed out of one Cyanobacterium affiliated with the genus *Synechococcus*, two Gammaproteobacteria with one related to the species *Moraxella osloensis*, and one related only to uncultured bacteria, one Betaproteobacterium related to the species *Acidovorax facilis*, one Alphaproteobacterium related to the species *Bosea vestrisii*, one member of the Planctomycetes and of the Bacteroidetes; both were not related to any described genus. In conclusion, the bacterial drinking water community was mostly composed of typical freshwater bacteria.

FACS analysis after viability staining allowed for more than 90% of the phylotypes detected in the (unsorted) drinking water samples a recovery in either the live or the dead sorted fraction, or in both fractions, i..e. only three phylotypes that usually had a very low abundance in the unsorted drinking water samples were not any more detected after FACS analysis. 32% of the phylotypes were only detected in the live sorted fraction, 21% in the dead sorted fraction, and 46% of the phylotypes were detected in both fractions. The Alphaproteobacteria and the Chloroflexi showed for all phylotypes live and dead sorted cells. The largest percentage of dead sorted phylotypes (i.e. no cells in the live sorted fraction detected) was observed for the Gammaproteobacteria (38%).

In general, our findings are consistent with those reported by other studies on drinking water. The fraction of membrane intact cells is comparable to observations by Berney et al. (3) for tap water free of chlorine, i.e. 66% live cells. The composition of the drinking water microflora on the phyla and class level compares well with studies on drinking water by Eichler et al. (8), and Hoefel et al. (10). Matthieu et al. (13) showed a comparable presence of Proteobacteria for drinking water, also for biofilm, and a pronounced response to chlorination.

The progress achieved by the here presented approach is that the species level and the species viability can be addressed. First, the species level allows a better insight into the changes of the bacterial community (e.g. Brettar et al.(6)). This can be of relevance for bacteria catalyzing specific processes, e.g. nitrifiers, and is of special relevance for the detection of pathogenic bacteria. Analysis up to the species level is a prerequisite for monitoring of potentially pathogenic species. In addition, the viability of the species is assessed what allows a better estimate of the risk for pathogenic bacteria, but can also be of interest with respect to bacterial species producing noxious substances or catalysing undesired processes.

2.5 Conclusion

The combined molecular analysis of the drinking water microflora revealed that a significant fraction of the bacteria in the drinking water was membrane-injured. Membrane injury is considered as an indicator of cell death, but it is still under debate if a bacterium is able to recover after membrane injury or not (7). So far published studies indicate that the membrane-injured bacteria detected by this staining technique can be considered as dead (3, 4, 10, 12). Since the aspect of cell death is of prime concern especially with respect to pathogenic bacteria in drinking water, it asks for further studies by a set of different approaches.

The study showed that bacterial species are differently affected by the drinking water treatment, disinfection and transport. This indicates the need of taxonomic analyses of the bacterial drinking water community for an assessment of the drinking water treatment process in order to achieve a complete elimination of pathogenic and potentially pathogenic bacteria from drinking water.

In conclusion, the applied approach enables monitoring of the bacterial drinking water community and assessment of the physiological state of relevant taxonomic groups, and can support the development of a more efficient and safer drinking water treatment, storage and distribution. A major advantage of the presented approach is that it allows an overview on the whole bacterial community; thus, also unexpected and potentially pathogenic bacteria can be detected (9), an important aspect especially in the light of climate change where hitherto unknown “emerging” pathogens are expected (17).

2.6 Acknowledgements

The technical support by Julia Strömpl is greatly acknowledged. This work was supported by funds from the European Commission for the HEALTHY WATER project (FOOD-CT-2006-036306). The authors are solely responsible for the content of this publication. It does not represent the opinion of the European Commission. The European Commission is not responsible for any use that might be made of data appearing therein.

2.7 References

1. **Alvarez-Barrientos A., J. Arroyo, R. Canton, C. Nombela, und M. Sanchez-Perez**. 2000. Applications of flow cytometry to clinical microbiology. Clin Microbiol Rev **13**:167-195.

2. **Berney M., F. Hammes, F. Bosshard, H. Weilenmann, und T. Egli**. 2007. Assessment and Interpretation of Bacterial Viability by Using the LIVE/DEAD BacLight Kit in Combination with Flow Cytometry. Appl. Environ. Microbiol. **73**:3283-3290.

3. **Berney M., M. Vital, I. Hülshoff, H. Weilenmann, T. Egli, und F. Hammes**. 2008. Rapid, cultivation-independent assessment of microbial viability in drinking water. Water Res **42**:4010-4018.

4. **Berney M., H. U. Weilenmann, und T. Egli**. 2006. Flow-cytometric study of vital cellular functions in *Escherichia coli* during solar disinfection (SODIS). Microbiology **152**:1719-1729.

5. **Brettar I., und M. G. Höfle**. 2008. Molecular assessment of bacterial pathogens - a contribution to drinking water safety. Curr Opin Biotechnol **19**:274-280.

6. **Brettar I., M. Labrenz, S. Flavier, J. Botel, H. Kuosa, R. Christen, und M. G. Hofle**. 2006. Identification of a Thiomicrospira denitrificans-Like Epsilonproteobacterium as a Catalyst for Autotrophic Denitrification in the Central Baltic Sea. Appl. Environ. Microbiol. **72**:1364-1372.

7. **Chilton P., N. S. Isaacs, P. Manas, und B. M. Mackey**. 2001. Biosynthetic requirements for the repair of membrane damage in pressure-treated *Escherichia coli*. Int J Food Microbiol **71**:101–104.

8. **Eichler S., R. Christen, C. Höltje, P. Westphal, J. Bötel, I. Brettar, A. Mehling, und M. G. Höfle**. 2006. Composition and Dynamics of Bacterial Communities of a Drinking Water Supply System as Assessed by RNA- and DNA-Based 16S rRNA Gene Fingerprinting. Appl. Environ. Microbiol. **72**:1858–1872.

9. **Henne K., L. Kahlisch, J. Draheim, I. Brettar, und M. G. Höfle**. 2008. Polyvalent fingerprint based molecular surveillance methods for drinking water supply systems. Water Science & Technology: Water Supply **8**:527-532.

10. **Hoefel D., P. Monis, W. Grooby, S. Andrews, und C. Saint**. 2005. Culture-independent techniques for rapid detection of bacteria associated with loss of chloramine residual in a drinking water system. Appl Environ Microbiol **71**:6479-6488.

11. **Huq A., I. N. Rivera, und R. R. Colwell**. 2000. Epidemiological significance of viable but nonculturable microorganisms, S. 301–323. *In* Nonculturable Microorganisms in the Environment.

12. **Joux F., und P. Lebaron**. 2000. Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. Microbes Infect **2**:1523-1535.

13. **Mathieu L., C. Bouteleux, S. Fass, E. Angel, und J. Block**. 2009. Reversible shift in the alpha-, beta - and gamma-proteobacteria populations of drinking water biofilms during discontinuous chlorination. Water Res **43**:3375-3386.

14. **Moreno Y., J. L. Alonso, S. Botella, M. A. Ferrús, und J. Hernández**. 2004. Survival and injury of *Arcobacter* after artificial inoculation into drinking water. Res Microbiol **155**:726-730.

15. **Nwachcuku N., und C. P. Gerba**. 2004. Emerging waterborne pathogens: can we kill them all? Curr Opin Biotechnol **15**:175-180.

16. **Schwieger F., und C. C. Tebbe**. 1998. A New Approach To Utilize PCR-Single-Strand-Conformation Polymorphism for 16S rRNA Gene-Based Microbial Community Analysis. Appl. Environ. Microbiol. **64**:4870-4876.

17. **WHO**: Emerging Issues in Water and Infectious Disease. WHO Press, World Health Organization, Geneva, Switzerland; 2003.

CHAPTER 3

Assessing the species composition of viable bacteria in drinking water using Fluorescence Activated Cell Sorting (FACS) and community fingerprinting

Leila Kahlisch[1], Karsten Henne[1], Lothar Gröbe[2], Ingrid Brettar[1] and Manfred G. Höfle[1]

[1]Dept. Vaccinology and Applied Microbiology, Helmholtz Center for Infection Research (HZI), Inhoffenstrasse 7, 38124 Braunschweig, Germany

[2] Department of Cell Biology, Helmholtz Center for Infection Research (HZI), Inhoffenstrasse 7, 38124 Braunschweig, Germany

CHAPTER 3 Assessing the species composition of viable bacteria in drinking water using Fluorescence Activated Cell Sorting (FACS) and community fingerprinting

3.1 Abstract

Drinking water safety asks for a comprehensive monitoring of the bacterial microflora present. Culture-based monitoring only addresses a few indicator bacteria and has to cope with viable but nonculturable (VBNC) bacteria. Molecular methods cannot distinguish membrane intact from membrane injured bacteria. For a comprehensive molecular monitoring we combined live/dead staining (Propidium iodide and SYTO9), Fluorescence Activated Cell Sorting (FACS) and community fingerprinting. Analysing a set of finished drinking water samples, live-dead staining revealed that about half of the bacteria in the tap water were alive. Molecular analysis using 16S rRNA and 16S rRNA gene-based single strand conformational (SSCP) fingerprints and sequencing of 16S rRNA amplicons of DNA and RNA extracted before and after sorting revealed: i) DNA- and RNA-based overall community fingerprints differed substantially, ii) the bacterial community retrieved from RNA and DNA reflected different bacterial species, i.e. phylotypes, (31 RNA-based phylotypes and 24 DNA-based phylotypes; only two common phylotypes), iii) the retrieved species were primarily of aquatic origin, and iv) the fraction of phylotypes showing only membrane injured cells, membrane intact cells and both was comparable for RNA- and DNA-based analyses. We showed that DNA- and RNA- based molecular analysis are needed because i) more and different species are detected and ii) the part of the bacterial community showing higher overall variability is reflected by the RNA-based analysis. We conclude that our approach allows the distinction of live/dead bacteria on the species level with the perspective of increased sensitivity and adjustment towards target groups of interest.

3.2 Introduction

Drinking water commonly provides a diverse microflora to the end user despite water processing aiming at the elimination of microorganisms, as demonstrated by detailed molecular studies (14, 31). Bacteria originating from the source water, regrowth in bulk water and the biofilm of the distribution network contribute to the generation of a diverse bacterial community in drinking water (17). Molecular methods, such as 16S rRNA-based and 16S rRNA gene-based fingerprints, can provide an overview on the bacterial community and thus can overcome the restriction of cultivation based methods that detect only the few bacteria growing under the respective cultivation conditions (9). These molecular methods allow overcoming the problem of nonculturability for viable but

nonculturable (VNBC) bacteria, i.e. even under adequate cultivation conditions these bacteria do not grow due to physiological constraints (21). However, molecular methods based on 16S rRNA genes cannot distinguish between live and dead bacteria (7, 27). During the last years, a broad set of fluorescent stains was developed allowing insight into the physiological state of bacteria (22). Stains assessing membrane integrity, such as Propidium Iodide (PI) and SYTO9, are considered to distinguish between membrane intact and membrane injured cells (8). This staining procedure has been evaluated and compared by a set of studies to other staining procedures for assessment of the physiological state of the bacteria. Membrane injury was evaluated as a major criterion for cell death where recovery is highly unlikely (12, 22, 4).

Bacterial community fingerprints and subsequent sequencing of the single fingerprint bands followed by phylogenetic analysis provide an overview on the structure and composition of bacterial drinking water communities up to the species level (14). Single bacterial species can be detected by these fingerprints, e.g. from 16S rRNA-based Single Strand Conformational Polymorphism (SSCP) analysis, at a relative abundance of 0.1% and more using general bacterial 16S rRNA gene primers. Besides providing an overview, fingerprints allow the detailed study of any bacterial taxon in a community if specific primers are used to better understand its ecology (19). In addition, pathogenic bacteria, also unexpected ones, posing a health risk can be observed and identified without their prior anticipation.

16S rRNA-based fingerprint analyses can be based on the analysis of environmental DNA or RNA. In general, it is assumed that RNA-based fingerprints represent more the active part of the bacterial community and DNA-based analyses provide insight into the bacterial members present in the community (14). Since viability is a major issue for drinking water bacteria, the comparison of DNA- and RNA- based analyses is of great interest. Combining these DNA- and RNA-based fingerprint analyses with the distinction for membrane integrity could provide new insights in the bacterial microflora and its viability.

Today's drinking water quality assessment is still based on the culture-based detection of indicator bacteria, i. e. *Escherichia coli* or fecal enterococci. Though molecular methods could give a better insight into the bacterial community and increase safety of the drinking water, it is crucial to include the aspect of viability in the molecular methods used. To this end, we developed a procedure that combined the advantages of the culture independent view on the drinking water microflora by molecular methods and the discrimination of membrane intact and membrane injured cells provided by the viability stains. Using Fluorescence Activated Cell Sorting (FACS), the membrane intact ("live") and membrane injured cells ("dead") were separated and analyzed by community fingerprinting. The aim of our study was to elucidate by this approach which bacterial taxa

are alive in finished drinking water. Both nucleic acids, DNA and RNA, were extracted from the 3 fractions, i.e. total, “live” and “dead”, and analyzed by 16S rRNA-based and 16S rRNA gene-based SSCP fingerprinting followed by sequencing of the fingerprint bands to provide insight into the taxonomic composition of the bacterial community. The differences between DNA- and RNA-based fingerprints were analyzed with respect to gain information about the active part of the bacterial drinking water microflora, with the new aspect of membrane injury. To our knowledge, this is the first study that applies both, DNA- and RNA- based community analysis combined with live/dead staining.

3.3 Materials and Methods

3.3.1 Study site and sampling.

Drinking water samples were obtained on 3 days, i.e. 25 March 2008 (sampling A), 31 March 2008 (sampling B) and 5 May 2008 (sampling C) from the tap in lab D0.04 of the Helmholtz Centre for Infection Research (HZI), Braunschweig-Stöckheim, Germany. Sampling A and B were taken as samples where a high similarity was expected due to the short time interval, sampling C was considered to display a distinct community due to the previously observed seasonal changes (19). The drinking water originated from two surface water reservoirs (oligotrophic, and dystrophic water) situated in a mountain range 40 km south of Braunschweig. Water processing included flocculation/coagulation, sand filtration and chlorination (0.2 - 0.7 mg l^{-1}). In the year 2008, only chlorine concentrations under 0.02 mg/l (method: colorimetric test “Aquaquant Chlor” from Merck for detection of free and total chlorine, detection limit 0.01mg/l) were detected at the nearest sampling point upstream to the HZI by the local water supplier. More details on the respective drinking water supply system are given elsewhere (14).

For live/dead staining and fluorescence activated cell sorting (FACS), drinking water microorganisms were concentrated 100-400 fold. 18 liter of drinking water were filtered onto a 0.2 µm pore size polycarbonate filter (90-mm diameter; Nucleopore; Whatman, Maidstone, United Kingdom), scraped and washed off from the filter carefully with 25 ml of 0.9% NaCl in sterile water (Fig. 1). A part of the biomass was either immediately used for the staining procedure as indicated below, and an aliquot was immediately frozen for later molecular analysis (-70°C).

For comparing the impact of concentration on the drinking water microflora, the drinking water microorganisms were additionally harvested by our routine procedure, i.e. filtration of 5 liters of drinking water on a filter sandwich consisting of a 0.2 µm pore size polycarbonate filter (90 mm diameter; Nucleopore; Whatman, Maidstone, United Kingdom) with a precombusted glass fiber filter on top (90 mm diameter; GF/F; Whatman) according

to Eichler et al. (13). Filter sandwiches were stored at -70℃ until further analysis. Per sampling date, 5 sandwich filters were obtained.

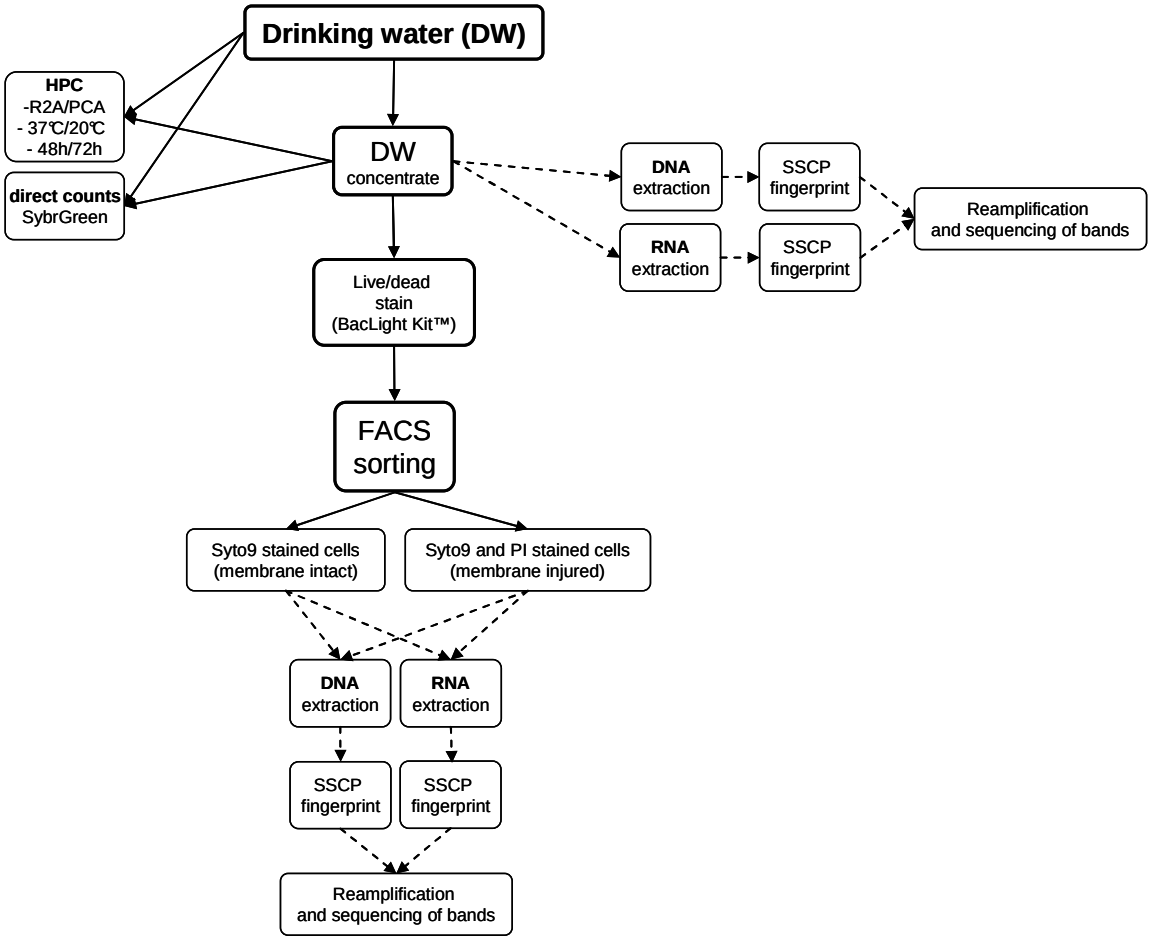


Fig. 1. Flow chart of the combined analysis of drinking water samples using FACS and SSCP fingerprinting. 18 liters of drinking water were filtered onto a 0.2µm Nucleopore filter, scraped and washed off the filter with 0.9 % saline solution. The drinking water bacteria were stained with the BACLight Kit™ for 20 min in the dark. After cell sorting, the differently stained fractions were analyzed by molecular methods (dashed lines), i.e. nucleic acids (DNA and RNA) were extracted and subjected to SSCP analysis. Sequence information was gained by reamplification and sequencing of single bands.

3.3.2 Staining and enumeration of drinking water bacteria.

Total bacteria from formaldehyde-fixed samples (2% final concentration) were stained with Sybr Green I dye (1:10000 final dilution; Molecular Probes, Invitrogen) for 15min at room temperature in the dark. Five ml portions were filtered onto 0.2 µm pore size Anodisc filters (Whatman) and mounted with Citifluor on microscopic glass slides according the protocol of Weinbauer et al. (32). Slides were either analyzed directly with epifluorescence microscopy or stored frozen (-20℃) until examination. For epifluorescence microscopy, a microscope (Axioplan, Zeiss) with suitable fluorescence filters was used and

the slides were examined using 100fold magnification. For each filter, either 10 photographs were taken and image sections of defined size (0.642mm x 0.483mm) were analyzed using the Image J software from MacBiophotonics (http://www.macbiophotonics.ca/) or 30 fields (0.125mm x 0.125mm) were counted by eye.

3.3.3 Heterotrophic plate counts (HPC).

HPCs were done in triplicate using an aliquot of the drinking water concentrate and the spread plate technique on either R2A agar (Oxoid) or tryptone soy agar (TSA; Oxoid) plates. Incubation was carried out at two different temperatures according to the German drinking water ordinance (36℃ for 48h and 22℃ for 72h) (1).

3.3.4 Concentrating, live/dead staining and FACS analysis of drinking water bacteria.

For fluorescence activated cell sorting (FACS), the concentrated biomass of the drinking water samples was stained for subsequent FACS analysis with SYTO 9 and propidium iodide (PI, final concentrations 5µM and 30µM, respectively; BacLight Kit, Molecular Probes (18)) according to the prescription of the manufacturer. After an incubation time of 20min in the dark, cells were subjected to FACS sorting using a MOFLO cytometer (Beckman Coulter, Krefeld, Germany) with a 488nm laser. The band pass filters used were 530/40nm and 616/26nm for SYTO 9 and PI, respectively.

3.3.5 Nucleic acid extraction from drinking water and sorted fractions.

DNA- and RNA- were extracted from the filter sandwiches and the concentrates of the drinking water samples; the latter were analyzed before and after staining and FACS-sorting as described above. For extraction of DNA and RNA, a modified DNeasy/RNeasy protocol (Qiagen, Hilden, Germany) was used. In this procedure, sandwich filters were cut into pieces, incubated with lysis buffer containing 10mg/ml lysozym (Sigma) for 30 min (DNA) or 20 min (RNA) in a 37℃ water bath. After a mechanical homogenization by shaking with glass beads the samples were heated to 70℃ in a water bath for 20min (DNA) or 15min (RNA). After filtration through a polyamide mesh with 250µm pore size, absolute ethanol was added to the filtrate (ratio filtrate/ethanol 2:1) and the mixture was applied to the adequate spin-column of the kit. After this step, the protocol was applied according to the manufacturer's instructions. For the RNA, a subsequent on-column DNase digestion was applied. Nucleic acids were eluted from the columns with DNase/RNase free water and stored at -20℃. The nucleic acids were quantified using Ribogreen (RNA or ssDNA quantification, Molecular Probes; Invitrogen) or Picogreen (dsDNA quantification, Molecular Probes; Invitrogen) according to Weinbauer and Höfle (33).

For extraction of the nucleic acids from the concentrated or the sorted fractions of microorganisms (considered as dead or alive), 1-2 ml portions of the concentrates before and after sorting were harvested by centrifugation for 15min at 15.000xg. The pellets were either frozen or directly used for nucleic acid extraction using the DNeasy/RNeasy protocol (Qiagen, Hilden; Germany). Pellet supernatant was checked by epifluorescence microscopy for microorganisms; in no case cells were observed. DNase digestion for the RNA was applied as described above.

3.3.6 16S rRNA and 16S rRNA gene based community fingerprints.

PCR amplification of 16S rRNA and of its respective genes from the extracted nucleic acids were performed using the previously described primers COM1 (5´-CAGCAGCCGCGGTAATAC-3´) and COM2 (5´-CCGTCAATTCCTTTGAGTTT-3´), amplifying positions 519 to 926 of the *Escherichia coli* numbering of the 16S rRNA gene (30). For single strand separation a 5´-biotin-labeled forward primer was used according to Eichler et al. (14). From RNA, a reverse transcription was carried out before PCR using the First strand cDNA synthesis Kit (Fermentas) following the manufacturer's recommendations. Each amplification was carried out using 2 ng DNA/cDNA template in a final volume of 50 µl, starting with an initial denaturation for 15 min at 95°C. A total of 30 cycles (30s at 95°C, 30s at 55°C, and 1 min at 72°C) was followed by a final elongation for 10 min at 72°C. Amplification was achieved using HotStar Taq DNA polymerase (QIAGEN, Hilden, Germany).

For the preparation of ssDNA and community fingerprints, a variant of the protocol described by Eichler et al. (14) was applied. Briefly, magnetic streptavidin coated beads (Promega, Madison, Wis.) were applied to obtain ssDNA from the PCR amplicons. Quantification of the obtained ssDNA was performed on a 1.5% agarose gel by comparison with a low-molecular-weight marker (Invitrogen low-DNA-mass ladder). For SSCP fingerprinting analysis, 25 ng of the obtained ssDNA was mixed with gel loading buffer (95% formamide, 10 mM NaOH, 0.25% bromphenol blue, 0.25% xylene cyanol) in a final volume of 7 µl. After incubation for 3 min at 95°C, the ssDNA samples were stored on ice, loaded onto a nondenaturing polyacrylamide-like gel (0.6x MDE gel solution; Cambrex BioScience, Rockland, Maine) and electrophoretically separated at 20°C at 400 V for 18 h on a Macrophor sequencing apparatus (Pharmacia Biotech, Germany). The gel was silver stained according to the method described by Bassam et al. (3). Dried SSCP gels were digitized using an Epson Expression 1600 Pro scanner, bands with an intensity of >0.1% of the total lane were considered for further statistical analysis. Similarity coefficients were calculated using Pearson correlation algorithm. Dendrograms were constructed using the

Unweighted Pair Group Method with Arithmetic mean (UPGMA) using the GelCompare II software (Applied Maths, Kortrijk, Belgium).

3.3.7 Reamplification and sequencing of ssDNA bands from SSCP fingerprints.

Sequence information was obtained following the protocol of Eichler et al. (14). Briefly, ssDNA bands were excised from the SSCP acrylamide gels, and boiled in Tris buffer (10 mM Tris-HCl, 5 mM MgCl2, 5 mM KCl, 0.1% Triton X-100, pH 9). 7µl of the solution was used in a reamplification PCR with the unbiotinylated COM primers described above. After checking the PCR-amplicons on a 2% agarose gel, the amplicons were purified and subsequently sequenced by cycle sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems, Foster City, Calif.). Before analysis on an ABI Prism 3100 Genetic Analyzer, the products were purified using the BigDye Terminator purification kit (QIAGEN). Phylogenetic identification of the sequences was done either by the NCBI Tool BLAST/blastn (2) for comparison with the closest 16S rRNA gene sequence or the Ribosomal Data Base Project Seqmatch Tool (11) for the identification of the closest described relative (Gene Bank Data base until September 9, 2009). To define a phylotype we chose two definite sequence differences on a mean stretch of 300bp sequence length as criterion. The partial 16S rRNA gene sequences retrieved from the fingerprints are accessible at the GenBank/EMBL/DDBJ accession numbers GQ 917122-GQ 9171174.

3.4 Results

3.4.1 Bacterial cell counts and heterotrophic plate counts.

The results on the bacterial counts are detailed in Figure 2. For drinking water samples obtained from the tap at the three sampling dates, the total bacterial cell numbers were in the range of 3 to 4 x 10^5 cells ml^{-1}; in the concentrates (100 to 400 fold) of the drinking water bacteria used for viability staining the cell numbers ranged from 5.1 x 10^7 to 1.2 x 10^8 cells ml^{-1}. After staining with PI and SYTO9, the fraction of membrane intact cells determined microscopically accounted for 53% ± 6% of the total bacteria while the membrane injured fraction accounted for 47% ± 6%. Heterotrophic plate counts (HPC) made from the concentrates were on average substantially less than the total bacterial counts, i.e. four to five orders of magnitude depending on medium and incubation time. Heterotrophic plate counts on R2A agar at 22℃ and after 72h exceeded all plate counts on the other media and temperatures, and ranged from 2.0 to 4.1 x 10^3 CFU ml^{-1} in the concentrate. For the unconcentrated tap water between 3.3 x 10^0 and 3.1 x 10^1 CFU ml^{-1} were detected.

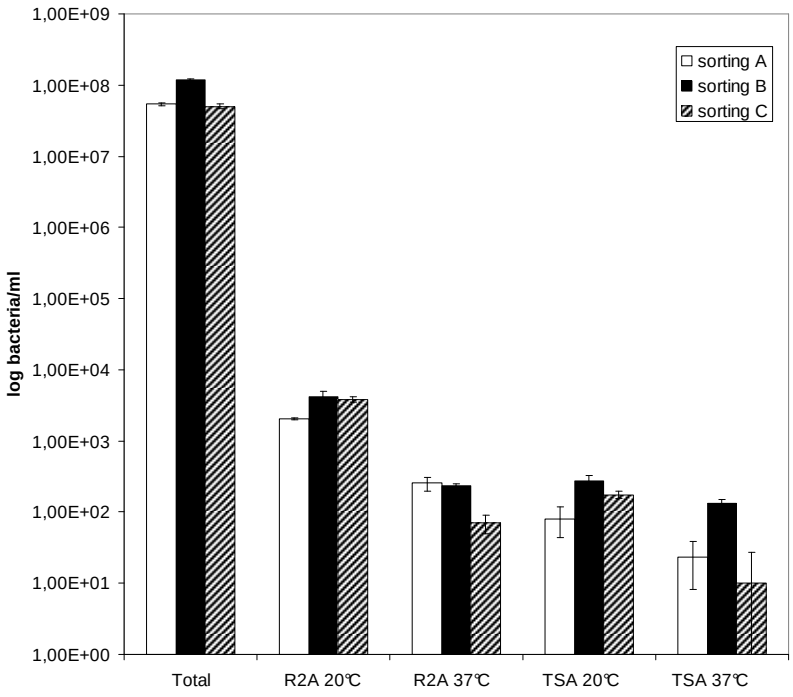


Fig. 2. Total bacterial cell numbers of the drinking water concentrate used in the three FACS sorting experiments. Sorting A (25.03.08, open bars) sorting B (31.03.08, black bars) and sorting C (05.05.2008, hatched bars). Total bacterial counts were determined by epifluorescence microscopy using Sybr Green I staining of formaldehyde fixed samples. Heterotrophic plate counts were determined using 1ml (or appropriate dilutions) concentrated drinking water and the spread plate technique on the media and temperatures indicated. Error bars represent standard deviation of at least 3 replicates.

3.4.2 FACS results of live/dead stained drinking water bacteria.

After live/dead staining, drinking water bacteria were analyzed based on two scatter parameters (forward and side scatter) and the fluorescence signal. For the analysis, some bacteria were excluded due to a lower forward scatter signal indicating cell debris with little or no DNA content (Fig. 3 a). On the basis of the two different stains, the majority (around 70-80%) of all cells could be sorted into two fractions, i.e. i) non membrane-injured SYTO9 positive cells and ii) membrane-injured or membrane injured PI positive cells (Fig. 3b).

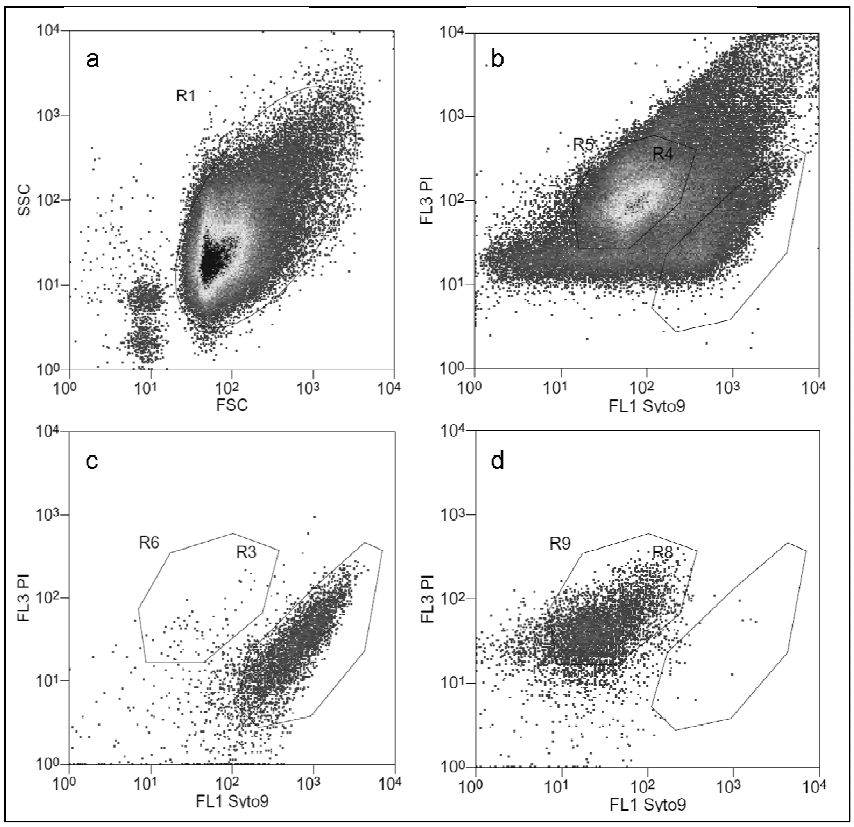


Fig. 3. Results of the FACS analysis of the drinking water community. Microorganisms from 18 liters of drinking water were concentrated, stained with the BacLight Kit™ and analyzed by the flow cytometer. (a) Flow cytometric analysis of unstained cells. Cells in gate R1 are included in the analysis and cells outside the gate were considered cell debris. (b) Flow cytometric analysis of microorganisms stained with the BacLight Kit™. Cells in gate R4 are SYTO 9 positive, cells in gate R5 are PI positive. Purity control of the sorted fractions: (c) SYTO 9 positive cells (gate R3) but PI negative (gate R6) and in (d) PI positive cells (gate R9) but negative for SYTO 9 (gate R8). Fluorescence channel: FL 1, 530±40nm; FL3, 616±16nm; FSC, forward scatter; SSC, side scatter.

Subsequent purity control as well as a check by epifluorescence microscopy demonstrated the effectiveness of the sorting (Fig. 3c and d). Flow cytometric analysis of the drinking water bacteria, based on comparison with reference beads of defined sizes, indicated that all fractions of microorganisms (total, SYTO9 positive, PI positive) had a

narrow size distribution and a rather small diameter, i.e. on the average 0.69µm (c_v:1.3%) (data not shown). In the three sorting experiments, total cell numbers recovered from FACS ranged around 10^6 cells per fraction (membrane intact, membrane injured) that were subsequently subjected to nucleic acid extraction and fingerprinting.

3.4.3 Structure of the bacterial community of drinking water before and after sorting

DNA- and RNA-based 16S rRNA SSCP fingerprints were used to analyze the bacterial community structure and composition of the drinking water before and after the cells were sorted by FACS as membrane intact and membrane injured cell fractions, and to assess the effect of the concentration procedure on the bacterial community (Fig. 4, 5). A general observation was that DNA- and RNA- based fingerprints from the same samples showed always very different banding patterns, a feature that was confirmed (see below) by the analysis of the species composition by sequencing of the fingerprint bands. DNA- and RNA-based SSCP fingerprints of the drinking water community with and without concentration (the latter sampled on filter sandwiches) showed very similar patterns as reflected in the cluster analysis by a similarity of always more than 80%, thus confirming the success of the concentration process necessary for FACS analysis (see suppl. Material Fig. 1). Fingerprints of the unsorted drinking water concentrates generated on the three sampling dates clustered closely together indicating a high similarity for the structure of the drinking water bacterial community on the three sampling dates (Fig. 5). As shown in Fig. 5a, the highest similarity was observed among sampling A and B for the DNA-based fingerprints (95%); the similarity of the concentrates was always higher than 76% irrespective of DNA- or RNA-based analyses or the sampling date (Fig. 5a,b, respectively).

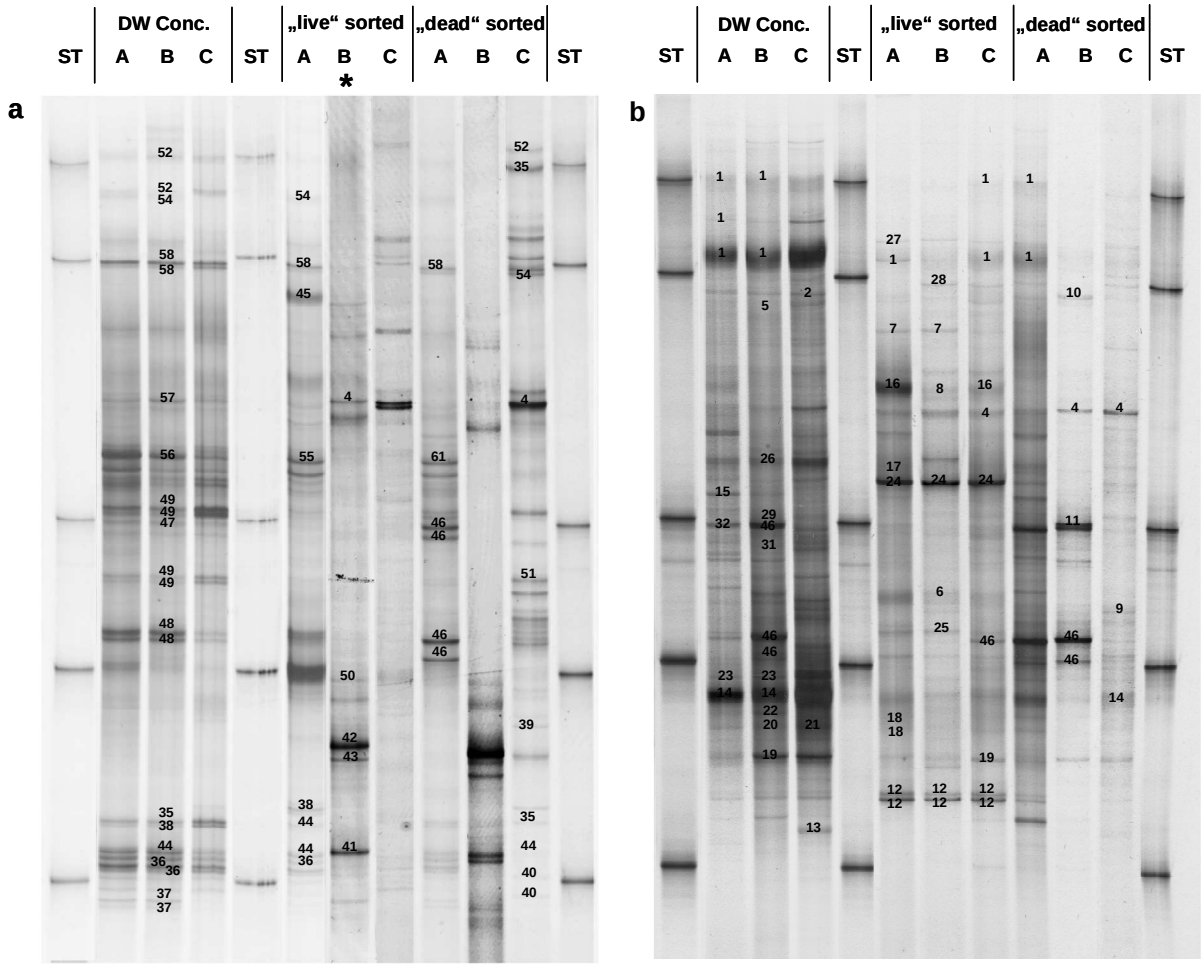


Fig. 4. (a) DNA-based 16S rRNA gene SSCP fingerprints of the different FACS sorting experiments: sorting A (25.03.08) sorting B (31.03.08) and sorting C (05.05.2008). Numbers represent single phylotypes of sequenced and identified bands. Phylogenetic information about these phylotypes is given in suppl. Table 1. Designations of single samples: ST, species standard; DW Conc., concentrated, unsorted drinking water samples from the respective dates; membrane intact ("live") sorted, SYTO9 positive fraction of the drinking water; membrane injured ("dead") sorted, propidium iodide positive fraction of the drinking water. The asterisk indicates a lane from a different SSCP gel. **(b) RNA-based 16S rRNA SSCP fingerprints of the different FACS sampling dates**. Sample designations and numbering of sequenced phylotypes are as for panel a. Phylogenetic information about the numbered phylotypes is given in suppl. Table 2.

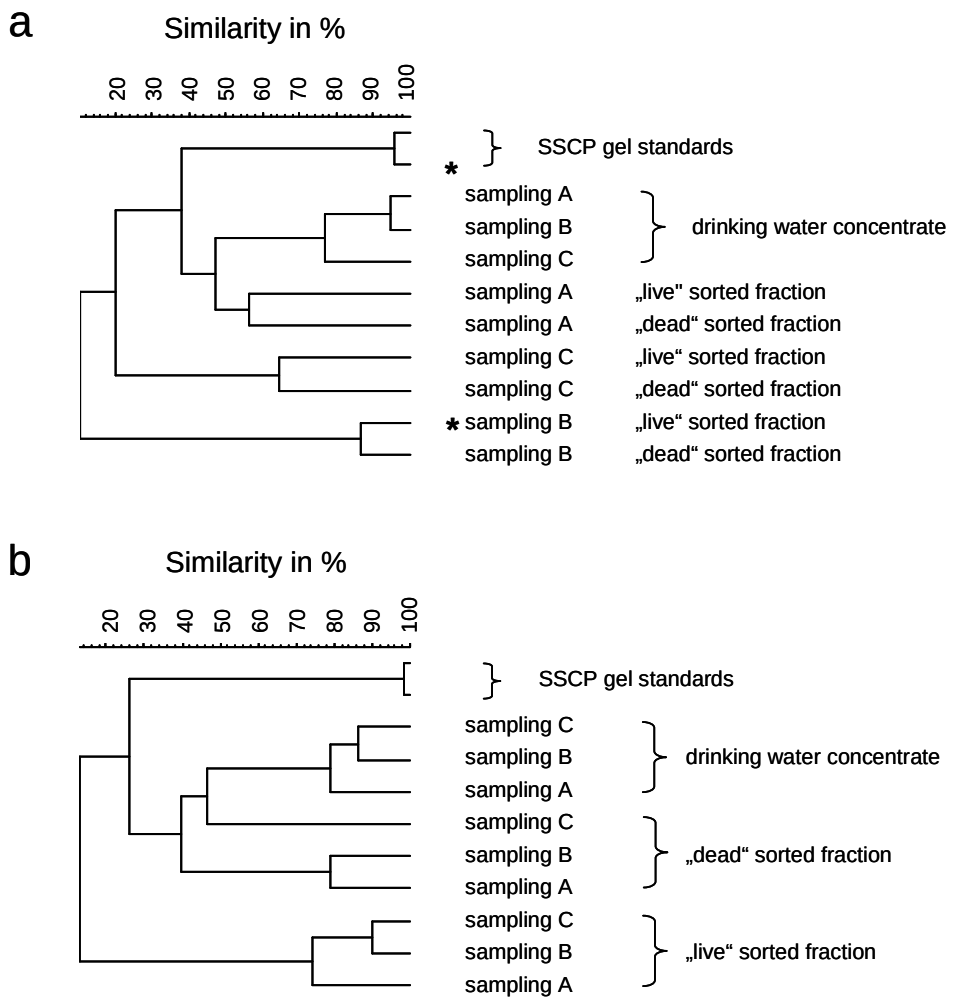


Fig. 5. Cluster analysis of the two SSCP gels given in Figure 4. Similarity coefficients were calculated using Pearson correlation algorithm. Dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA). **(a)** DNA-based SSCP fingerprints of the different FACS sampling dates: sorting A (25.03.08) sorting B (31.03.08) and sorting C (05.05.2008). Sample designations are as in Fig. 4a. The lane labeled with an asterisk is from a different SSCP gel. **(b)** RNA-based SSCP fingerprints of the different FACS sampling dates. Species standards were taken as out-group for the cluster analysis. Sample designations are as in Fig. 4b.

DNA-based fingerprints of the membrane intact and membrane injured sorted fractions showed a very distinct pattern for each sampling day (Fig. 4a). Comparative cluster analysis of the DNA-based fingerprints showed that for each sampling date the fingerprints from the membrane intact and membrane injured sorted cell fractions clustered more closely together than the different sampling dates (Fig. 5a), indicating that the community structure became more dissimilar among the sampling dates due to the live/dead sorting. RNA-based fingerprints of the membrane intact and membrane injured sorted cell fractions showed a similar pattern among the membrane intact sorted fractions irrespective of the sampling date (Fig. 4b) as indicated by a tight clustering (similarity >70%, Fig. 5b). The membrane injured sorted fractions showed a more diverse pattern for the three sampling dates, mainly caused by the large discrepancy for sampling C. If the membrane injured sorted fraction of sampling C is neglected, the general observation was that the samples had a similar but distinct pattern for each sampling day, i.e. the community structure showed a comparable level of similarity before and after sorting among the different sampling dates.

3.4.4 Taxonomic composition of the different cell fractions.

A total of 111 bands from the DNA- and RNA-based SSCP fingerprints were sequenced to determine the taxonomic composition of the different fractions. Using a limit of ≥ 99% 16S rRNA gene sequence similarity as discrimination criterion, we retrieved 53 unique phylotypes for these bands (suppl. Material Table 1 and 2). For identification, the obtained sequences were compared to all databank entries in the GeneBank. Out of the 53 unique phylotypes, 31 were retrieved from the RNA-based fingerprints, and 24 from the DNA-based fingerprints with only two phylotypes that were retrieved from both RNA and DNA. RNA-phylotype 1 and DNA-phylotype 52 were affiliated with the same species but were distinct by 8 nt and therefore assigned to different phylotypes. Thus, the bacterial community reflected by both fingerprint types differed to a large extent.

Comparing the major taxonomic groups, the analysis of the DNA-based fingerprints (Fig. 6a) showed that the drinking water samples were dominated by members of the *Betaproteobacteria* (8 phylotypes, with an average abundance of 15%), *Bacteroidetes* (7 phylotypes, 17.8%), and *Actinobacteria* (2 phylotypes, 15.3%). All other classes and phyla, i.e. *Alpha*- and *Gammaproteobacteria*,

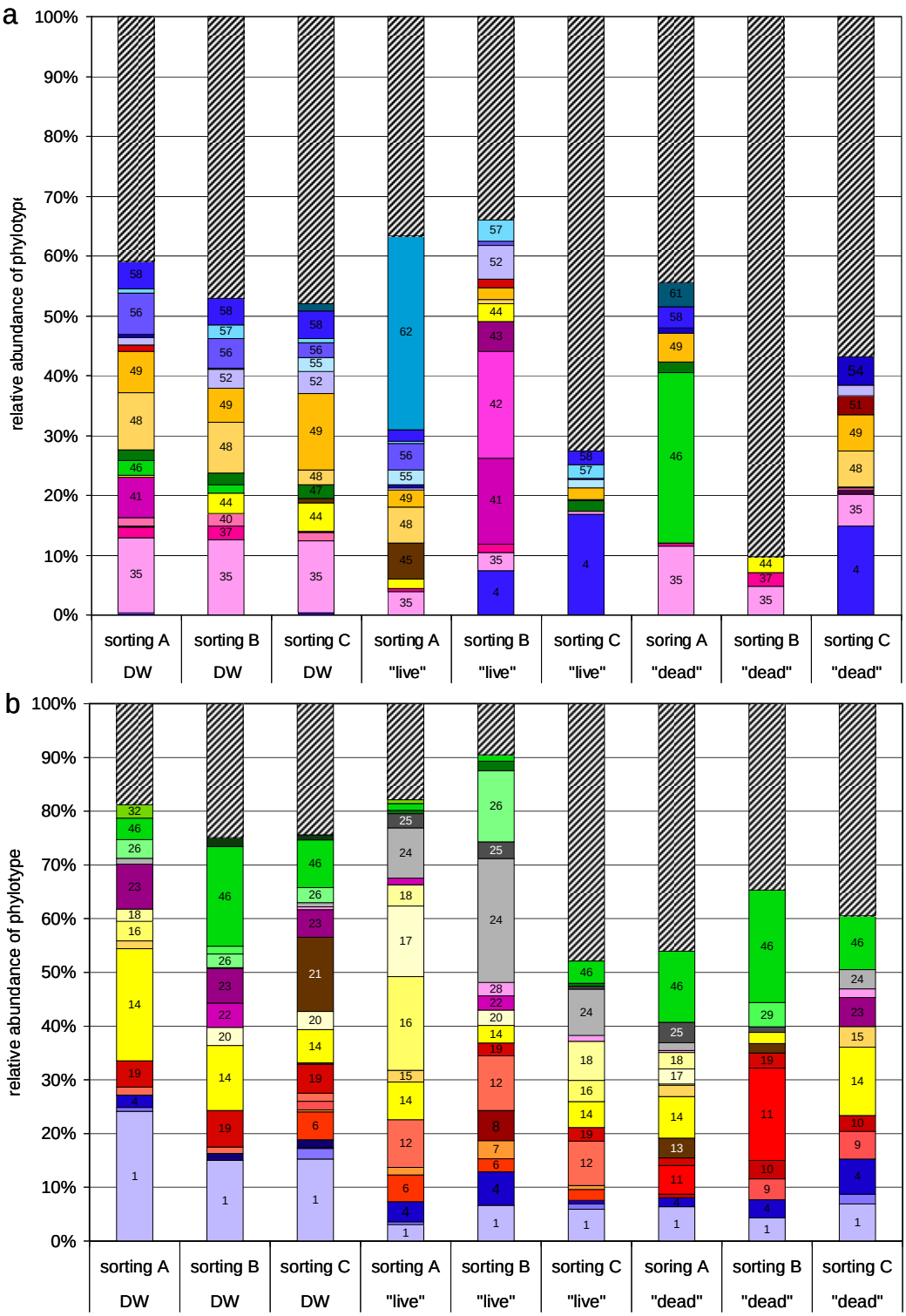


Fig. 6. Comparison of relative abundances of the phylotypes found in the different cell fractions and the drinking water concentrate (DW) on the three different sampling dates. (a) Phylotypes from the DNA-based SSCP fingerprints. (b) Phylotypes from the RNA-based SSCP fingerprints. Numbers represent the single phylotypes given in suppl. Table 1 and 2, respectively. The colors are corresponding to the major phylogenetic groups of the phylotypes: Yellow – *Alphaproteobacteria*; Blue – *Betaproteobacteria*; Red – *Gammaproteobacteria*; Green – *Cyanobacteria*; Violet – *Bacteriodetes*; Brown – *Planctomycetes*; Orange – *Actinobacteria*; Grey – *Chloroflexi*. Hatched bars represent unidentified bands.

Planctomycetes and *Cyanobacteria*, had a low diversity (1-2 phylotypes) and a low abundance (0.2 -3.3%). The RNA-based fingerprints (Fig. 6b) of the drinking water samples were dominated by members of the *Betaproteobacteria* (4 phylotypes, 22.8%), *Cyanobacteria* (6 phylotypes, 15.6%), *Alphaproteobacteria* (5 phylotypes, 15.6%), *Gammaproteobacteria* (8 phylotypes, 9.5%), and *Bacteroidetes* (3 phylotypes, 8.3%). The remaining 4 phyla, i. e. *Nitrospira, Firmicutis, Planctomycetes, Chloroflexi*, had a low diversity (1-2 phylotypes) and a low abundance (0.1-4.6%). Most striking was the pronounced discrepancy between phylotypes of the RNA and DNA-based analyses with only two phylotypes (phylotype 4 and 46) retrieved from both analyses. While most phyla occurred in both the RNA- and DNA-based analyses, *Actinobacteria* (with a high abundance in the DNA-based analyses, i.e. up to 10% for phylotype 48, and up to 13% for phylotype 49), were never observed in the RNA-based analyses, whereas *Chloroflexi* (phylotype 24 with a high abundance up to 23% in the membrane intact sorted fraction of the RNA-based analyses) were never observed in the DNA-based analyses. The single phylotypes of *Nitrospira* and *Firmicutes* also occurred only in the RNA-based analyses but had low and variable abundances (below 4%). Phylogenetic analysis of the retrieved phylotypes together with the nearest cultured species can be followed precisely in supplementary figure 3a (all occurring phyla) and b (phylum *Proteobacteria* in detail).

For an estimate of the origin of the phylotypes, the habitat of the most similar bacterial sequence from the public data bases is given in supplementary Table 1 and 2. Provided that the most similar sequence i) had a similarity of higher or equal to 91% 16S rRNA gene similarity and ii) was of aquatic origin, the phylotype was rated as “of aquatic origin”. Below 91% 16S rRNA gene sequence similarity the relatedness was regarded as too low to give information on the potential habitat of the phylotype. Based on these criteria, 76% of the DNA and the RNA-based phylotypes were considered as of aquatic origin which most of them from freshwater habitats. Six out of the RNA phylotypes and three out of the DNA phylotypes were not used for this assignment due to too low sequence similarity (all below 88% to the next sequence in the public data bases).

All 24 DNA-based phylotypes were recovered after cell sorting in the membrane intact and/or membrane injured fractions indicating a recovery of 100% of the phylotypes in the sorted fractions. 38% of the DNA-phylotypes occurred only in the membrane intact fraction, 21% occurred only in the membrane injured fraction, and 42% occurred in both fractions. Phylotypes of the major taxa *Betaproteobacteria* and *Bacteroidetes* contributed to all three fractions, i.e. membrane intact, membrane injured and total. The two phylotypes of the *Actinobacteria* were always retrieved from the membrane intact and membrane injured fractions. Based on the RNA analyses, 28 of the 31 phylotypes (90%) were retrieved after sorting in the membrane intact and/or membrane injured fraction. From the

retrieved 28 phylotypes, 32% of the RNA-phylotypes occurred only in the membrane intact fraction, 21% occurred only in the membrane injured fraction, 46% occurred in both fractions. Phylotypes of the classes *Gammaproteobacteria*, *Cyanobacteria* and the phylum *Bacteroidetes* contributed to all three fractions, i.e. membrane intact, membrane injured and total. All phylotypes of the *Alphaproteobacteria* were always retrieved from membrane intact and membrane injured fractions. Thus, RNA- and DNA-based analyses showed a similar ratio for the phylotypes with respect to retrieval from the membrane intact and membrane injured fractions: 32-38% in the membrane intact fractions, 21% in the membrane injured fractions, and 42%-46% in both fractions.

From the 24 phylotypes of the DNA analyses, five phylotypes contributed to more than 5% (up to 13%) of the total, unsorted drinking water community. Three of these five dominating phylotypes were not related to any cultured species or described genus (below 93% 16S rRNA similarity) namely two *Actinobacteria* (phylotype 48, 49), and one member of the *Bacteroidetes* (phylotype 41) The other two showed both 98% similarity to cultured species, in detail one member of the *Bacteriodetes* (phylotype 35) and one betaproteobacterium (phylotype 56). From the 31 phylotypes of the RNA analyses, seven phylotypes contributed to more than 5% (up to 24%) of the total (unsorted) drinking water community. These eight dominating phylotypes were composed of one cyanobaterium (phylotype 46; affiliated with the genus *Synechococcus*), two *Gammaproteobacteria* with one related to the genus *Moraxella* (phylotype 6), and one related only to uncultured bacteria (phylotype 19), one betaproteobacterium related to the species *Acidovorax facilis* (phylotype 1), one alphaproteobacterium related to the species *Bosea vestrii* (phylotype 14), one member of the *Planctomycetes* (phylotype 21) and of the *Bacteroidetes* (phylotype 23); both were not related to any described genus.

After FACS-sorting, major changes of the abundances of the phylotypes occurred that were far more pronounced for the DNA-based analyses than for the RNA-based analyses. Supplementary Fig. 2 is providing more details on the changes of abundances before and after sorting. These changes of abundances through sorting was most pronounced in the membrane intact sorted fraction for the *Chloroflexi* (PT 24) in the RNA-based analyses and the *Planctomyces* (no. 62) in the DNA- based analyses. Overall, we observed only few phylotypes with a high abundance in the sorted cell fractions of the DNA-based electropherograms (suppl. Material Fig. 2a) while in the RNA-based electropherograms (suppl. Material Fig. 2b) phylotypes with a high abundance were present in the non-sorted as well as in the sorted fractions.

Overall, the bacterial drinking water community retrieved from RNA and DNA analyses was mostly composed of bacteria that were not related to any described species. For the DNA-based analyses 46% of the phylotypes were not related to any described

genus, 42% were affiliated with a described genus, and 38% were affiliated with a described species. For RNA-based analyses 58% of the phylotypes were not related to any described genus, 32% were affiliated with a described genus, and 23% were affiliated with a described species. The phylotypes affiliated with a described genus were mostly members of the *Bacteroidetes, Alpha-, Beta-* and *Gammaproteobacteria*. Most of the phylotypes had a high similarity with 16S rRNA gene sequences of aquatic origin, predominantly freshwater.

3.5 Discussion

3.5.1 Community structure and composition of drinking water bacteria using DNA- and RNA-based fingerprints.

DNA- and RNA-based molecular analyses provided a very different picture of the drinking water microflora. This comprised the overall fingerprint patterns, their changes due to sorting and the retrieved phylotypes. However, this overview does not precisely reflect the quantitative composition of the bacterial community. Since the amplification of 16S rRNA genes is based on PCR, a PCR bias has to be taken into account (16, 35). According to our experience with aquatic community analysis by SSCP, the technique provides highly reproducible fingerprints of the community with high reproducibility in terms of the relative abundances of the single bands compared to the total community. Compared to real-time PCR detection of single phylotypes, low abundant phylotypes seem to be overestimated, while highly abundant phylotypes seem to be underestimated (10). Thus, the fingerprint gives a biased but reproducible quantitative picture of the bacterial community allowing comparison of different bacterial communities and understanding of the dynamics of single community members.

The fingerprint analysis of the drinking water samples showed a highly consistent pattern among the three different sampling dates for both the RNA- and DNA-based analyses. A rather stable bacterial community of the investigated drinking water over time had already been shown by the seasonal study of Henne et al. (19) using DNA-based fingerprints. Though seasonal variation occurred for some members of the bacterial community, the overall community structure was rather stable during the year. The SSCP fingerprint patterns were completely different with respect to analysis of RNA and DNA of the same samples. This different pattern was confirmed by sequencing and phylogenetic analysis of the fingerprint bands. From the 24 phylotypes retrieved from the DNA-based analysis, and 31 phylotypes retrieved from the RNA-based analysis only two phylotypes (PT 4, 46) were identical, and two were affiliated with the same species (PT 1, 52). Though the same phyla with a few exceptions were detected in RNA and DNA-based analysis, from the genus level upwards there was a pronounced divergence. A strong discrepancy

between RNA and DNA-based analysis concerning the fingerprint pattern and the members of the bacterial community was also observed by Eichler et al. (14).

Our drinking water community was dominated by phyla and classes typical for freshwater environments, i.e. *Bacteroidetes, Cyanobacteria, Betaproteobacteria* and *Gammaproteobacteria*. This was also the case when looking at the higher level of phylogenetic resolution, i.e. the phylotypes that were resolved at the species level. The majority of the phylotypes (76%) were most closely related to sequences retrieved from aquatic habitats. This is consistent with findings of the study of the whole drinking water supply system by Eichler et al. (14). The phylotypes identified based on the DNA-based molecular analyses seemed to have a higher stability in the drinking water than the RNA phylotypes. 55% of the phylotypes identified in this study were also detected in the study of Eichler et al (14) in the same drinking water supply system 5 years ago. This was different for the RNA-based phylotypes that had only a reoccurrence of 11%.

3.5.2 Assessment of live and dead bacterial cells using PI/SYTO9 staining.

In our study about half (53%) of the bacterial cells in the drinking water samples showed an intact membrane. This is in line with studies by Berney et al. (5) that reported a fraction of membrane intact cells of about 66% in tap water that was free of chlorine as it was the case in our study. For chlorine containing tap water, Hoefel et al. (20) reported 12% membrane intact cells for finished drinking water of an Australian water distribution system with a higher chlorination during treatment and transport, and free a chlorine residual level at the tap of 0.4 mg l^{-1}.

The Propidium Iodide staining is considered to provide a good estimate for membrane injury of *Bacteria* and *Archaea* (25). In a set of studies, this staining procedure has been evaluated and compared with other staining procedures for assessment of the physiological state of the bacteria (15, 22). Besides the evaluation of methodological aspects, recently studies were done for drinking water with added bacteria and the indigenous microflora. Berney et al. (6) tested PI for *E. coli* in drinking water submitted to UV and sunlight irradiation using a set of different viability stains. The study showed that loss of membrane integrity as indicated by PI staining was the final signal after decrease of all other tested physiological functions. In a second study, Berney et al. (5) used PI staining for analyzing the microflora of a set of drinking water samples. The viability of the drinking water bacteria was higher for the bottled water (about 90%) and the drinking fountain water (about 85%) than for the drinking water at the tap (about 66%). The high percentage of viable cells coincided with a high ATP content. The comparison of PI staining with other methods demonstrated PI staining was a valuable criterion for live-dead distinction for drinking water bacteria.

Autofluorescence is a feature that has to be taken into account as a potentially misleading signal for the analysis of aquatic bacterial communities by PI/SYTO9 staining (36). According to our taxonomic analyses, two phylotypes were affiliated with the phylum *Chloroflexi* whose members are known to contain bacteriochlorophyll c and a in the chlorosomes and the cytoplasmic membrane resulting in green autofluorescence (26). The *Chloroflexi* were detected in the membrane intact and membrane injured sorted fractions, but with a far higher detection in the membrane intact fractions (up to 23% for PT 24 in the RNA-based analyses). In the latter case a wrong “live” sorting due to the autofluorescence cannot be ruled out. On the other hand, a false “dead” sorting could have been caused by phylotypes affiliated with the genus *Synechococcus* due to the presence of red fluorescent phycoerythrin (34). Phylotype 46 that was common in the RNA and DNA-based analyses and closely related to *Synechococcus rubescens* had a very high “dead”- sorting for up to 29% of the total membrane injured sorted fraction for the DNA and up to 21% for the RNA analysis, respectively. Though autofluorescence may be misleading for the live-dead sorting of some bacteria with photosynthetic pigments, we do not consider this as a critical issue for the live/dead staining procedure as a distinction for drinking water bacteria. Autofluorescent bacteria are commonly not considered as pathogenic because these bacteria are all heterotrophic and therefore, autofluorescence does not seem a critical issue for our staining procedure in terms of public health.

3.5.3 Live and dead assessment of different phyla and phylotypes

All DNA-based phylotypes and 90% of the RNA-based phylotypes were retrieved after sorting in the membrane intact and/or membrane injured fraction. The three missing phylotpes might have been missed due to their low abundance. This close to complete recovery of the phylotypes after sorting allows a comparison of the sorting results between the DNA- and RNA- based analyses. Though the sequencing success was 77% for the RNA-based analyses, and only 57% for the DNA-based analyses, the comparison can be done on the level of the retrieved phylotypes that indeed had a relatively high abundance compared to the not retrieved phylotypes.

A comparison shows that the phylotypes reflected by the DNA-based analyses have the same size of the “dead fraction” as those reflected by the RNA-based analyses, i.e. 21%. Also, the DNA- and RNA- phylotypes had a comparable percentage of only membrane intact sorted (DNA, 38%; RNA, 32%) and of both membrane intact and membrane injured sorted phylotypes (DNA: 42%, RNA, 46%). Phylotype 4 commonly retrieved from DNA- and RNA- analyses was recovered from membrane intact and membrane injured fractions in the DNA- and RNA-based analysis, i.e. for the only common phylotype comparable sorting results were obtained for the DNA and RNA-based analysis.

The second common phylotype (PT 46) cannot be compared due to the potential interference with the pigments (see above). Based on our observation, we can say that the fraction of membrane injured phylotypes is not higher for the bacteria reflected by the DNA analyses than those of the RNA analyses. This is an essential finding because it was often speculated that those reflected by the RNA are alive, and those reflected by the DNA are dead (14). Based on this observation, we assume that the reason for the detection of a phylotype in the DNA- or RNA-based analyses might be the phylotype-specific regulation of the DNA and the RNA pool and was obviously not related to the viability of the respective phylotypes. This is consistent with analyses of Klappenbach et al. (23) showing a broad range of numbers of rRNA operons (1-13) specific for each bacterial strain. Furthermore, the value of adding RNA-based analyses to the DNA-based analyses was demonstrated because many bacterial species - including some with pathogenic potential - are missed when only the common DNA-based analyses are performed.

3.5.4 Taxonomic composition of the bacterial community of drinking water and human health

The bacterial community was composed of seven phyla (see suppl. Material Table 1 and 2). The phyla as well as the phylotypes are primarily those typically present in aquatic ecosystems (14, 37). However, some of the phylotypes detected in the drinking water have the potential of being opportunistic pathogens. The alphaproteobacterium PT 14 identified as closely related to *Bosea vestrii* in the RNA-based analysis was retrieved from the membrane intact and membrane injured sorted fraction, and was present in the drinking water at high abundances (6-21%). In addition, the betaproteobacterium PT 53 identified as *Achromobacter xylosoxidans* was detected, but in the membrane injured sorted fraction of the DNA-based analysis. Both species were occasionally associated with infections of immuocompromised people (24, 29).

Presence, viability and infectivity of pathogenic bacteria in drinking water are criteria that have to be fulfilled for posing a threat to human health. Presence of bacteria can be assessed by the applied technology to the detection limit of the method which is about 0.1% of the total microflora. Viability was assessed by the live/dead staining. Infectivity asks for the following: i) the precise of the taxonomic identification of the pathogen, and ii) a separate, mostly experimental, assessment of infectivity that has to be achieved in addition to molecular analyses. Concerning the precise assessment of the taxonomy, the about 400nt long sequences obtained from a SSCP gel can resolve, at best, the species level. This might be enough for environmental species that often have no closely related cultured neighbor species. For most pathogenic species, a full (>1400nt) 16S rRNA sequence is needed or even the sequence of other genes associated with infectivity of the

respective species, e.g. the *mip* gene for *Legionella pneumophila*. Thus, the proposed technology can provide a valuable monitoring tool that can show that a potentially harmful species is present - but it remains with the “potential” and the true risk has to be assessed consecutively by additional adequate measurements.

In conclusion, the approach used in this study is considered a valuable tool for drinking water monitoring. The applied PI/SYTO9 staining procedure indicating membrane injury of the bacterial cells is considered as a reliable criterion for damaged or dead bacterial cells. This is especially of value for monitoring of bacteria relevant to human health. The combined approach of DNA- and RNA-based fingerprint analyses with live-dead staining and sorting was demonstrated as a straight forward monitoring tool. This tool still can be modified and extended with respect to sensitivity or methodological details. For example, in terms of methodology, PI/SYTO9 stain could be replaced by propidium monoazide application followed by molecular analyses thereby avoiding the step of FACS sorting (28). For increased sensitivity with respect to specific groups of pathogenic relevance, the general bacterial primers (COM1, 2) could be replaced by group specific primer reaching a lower detection limit and a better taxonomic resolution of the targeted group. Therefore, further studies are needed to elucidate a potential health threat of drinking water with respect to specific species.

3.6 Acknowledgments

This work was supported by funds from the European Commission for the HEALTHY WATER project (FOOD-CT-2006-036306). The authors are solely responsible for the content of this publication. It does not represent the opinion of the European Commission. The European Commission is not responsible for any use that might be made of data appearing therein. We thank Josefin Draheim for outstanding technical support.

3.7 References

1. 2001. Verordnung über die Qualität von Wasser für den menschlichen Gebrauch (Trinkwasserverordnung - TrinkwV 2001) Geändert durch Art. 363 V v. 31.10.2006 I 2407.

2. **Altschul S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman**. 1990. Basic local alignment search tool. J Mol Biol **215**:403-10.

3. **Bassam B. J., G. Caetano-Anoll, and P. M. Gresshoff**. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem **196**:80-83.

4. **Berney M., F. Hammes, F. Bosshard, H. Weilenmann, and T. Egli**. 2007. Assessment and Interpretation of Bacterial Viability by Using the LIVE/DEAD BacLight Kit in Combination with Flow Cytometry. Appl. Environ. Microbiol. **73**:3283-3290.

5. **Berney M., M. Vital, I. Hülshoff, H. Weilenmann, T. Egli, and F. Hammes**. 2008. Rapid, cultivation-independent assessment of microbial viability in drinking water. Water Res **42**:4010-4018.

6. **Berney M., H. U. Weilenmann, and T. Egli**. 2006. Flow-cytometric study of vital cellular functions in *Escherichia coli* during solar disinfection (SODIS). Microbiology **152**:1719-1729.

7. **Birch L., C. Dawson, J. Cornett, and J. Keer**. 2001. A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. Lett Appl Microbiol **33**:296-301.

8. **Boulos L., M. Prevost, B. Barbeau, J. Coallier, and R. Desjardins**. 1999. LIVE/DEAD BacLight : application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J Microbiol Methods **37**:77-86.

9. **Brettar I., and M. G. Höfle**. 2008. Molecular assessment of bacterial pathogens - a contribution to drinking water safety. Curr Opin Biotechnol **19**:274-280.

10. **Brettar I., M. Labrenz, S. Flavier, J. Botel, H. Kuosa, R. Christen, and M. G. Hofle**. 2006. Identification of a *Thiomicrospira denitrificans*-Like Epsilonproteobacterium as a Catalyst for Autotrophic Denitrification in the Central Baltic Sea. Appl. Environ. Microbiol. **72**:1364-1372.

11. **Cole J. R., B. Chai, R. J. Farris, Q. Wang, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, A. M. Bandela, E. Cardenas, G. M. Garrity, and J. M. Tiedje**. 2007. The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. Nucl. Acids Res. **35**:169-172.

12. **Czechowska K., D. R. Johnson, and J. R. V. D. Meer**. 2008. Use of flow cytometric methods for single-cell analysis in environmental microbiology. Curr. Opin. Microbiol **11**:205-212.

13. **Eichler S., M. G. Weinbauer, D. Dominik, and M. Höfle**. 2004. Extraction of total RNA and DNA from bacterioplankton, chapter 1.0.8, S. 103-120. *In* G.A.Kowalchuk; F.J.D.Bruijn; I.M.Head; A.D.L.Akkermans; and J.D.van Elsas (Ed.), Molecular microbial ecology manual, 2andefined Aufl. Kluwer Academic Publishers, Dordrecht, The Netherlands.

14. **Eichler S., R. Christen, C. Höltje, P. Westphal, J. Bötel, I. Brettar, A. Mehling, and M. G. Höfle**. 2006. Composition and Dynamics of Bacterial Communities of a Drinking Water Supply System as Assessed by RNA- and DNA-Based 16S rRNA Gene Fingerprinting. Appl Environ Microbiol **72**:1858–1872.

15. **Falcioni T., S. Papa, and J. M. Gasol**. 2008. Evaluating the flow-cytometric nucleic acid double-staining protocol in realistic situations of planktonic bacterial death. Appl Environ Microbiol **74**:1767.

16. **Farrelly V., F. Rainey, and E. Stackebrandt**. 1995. Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl. Environ. Microbiol. **61**:2798-2801.

17. **Hammes F., M. Berney, Y. Wang, M. Vital, O. Köster, and T. Egli**. 2008. Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water Res **42**:269-277.

18. **Haugland R.** The Handbook—A Guide to Fluorescent Probes and Labeling Technologies. http://www.invitrogen.com.

19. **Henne K, Kahlisch L, Draheim J, Brettar I, and Hofle M**. 2008. Polyvalent fingerprint based molecular surveillance methods for drinking water supply systems. Water Science & Technology: Water Supply **8**:527-532.

20. **Hoefel D., P. Monis, W. Grooby, S. Andrews, and C. Saint**. 2005. Profiling bacterial survival through a water treatment process and subsequent distribution system. J Appl Microbiol **99**:175-186.

21. **Huq A., I. Rivera, and RR Colwell**. 2000. Epidemiological significance of viable but non culturable microorganisms., S. 301-323. *In* R. Colwell, and D.J. Grimes (Ed.), Nonculturable Microorganisms in the Environment (new edition). ASM press

22. **Joux F., and P. Lebaron**. 2000. Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. Microbes. Infect. **2**:1523-1535.

23. **Klappenbach J. A., J. M. Dunbar, and T. M. Schmidt**. 2000. rRNA Operon Copy Number Reflects Ecological Strategies of Bacteria. Appl. Environ. Microbiol. **66**:1328-1333.

24. **La Scola B., M. Mallet, P. A. D. Grimont, and D. Raoult**. 2003. *Bosea eneae* sp. nov., *Bosea massiliensis* sp. nov. and *Bosea vestrisii* sp. nov., isolated from hospital water supplies, and emendation of the genus Bosea (Das et al. 1996). Int J Syst Evol Microbiol **53**:15-20.

25. **Leuko S., A. Legat, S. Fendrihan, and H. Stan-Lotter**. 2004. Evaluation of the LIVE/DEAD BacLight Kit for Detection of Extremophilic Archaea and Visualization of Microorganisms in Environmental Hypersaline Samples. Appl. Environ. Microbiol. **70**:6884-6886.

26. **Madigan M. T., J. Martinko, and J. Parker**. 2002. Brock Biology of Microorganisms, 10th and defined Edition, Benjamin Cummings.

27. **Moreno Y., J. L. Alonso, S. Botella, M. A. Ferrús, and J. Hernández**. 2004. Survival and injury of *Arcobacter* after artificial inoculation into drinking water. Res Microbiol **155**:726-730.

28. **Nocker A., P. Sossa-Fernandez, M. D. Burr, and A. K. Camper**. 2007. Use of Propidium Monoazide for Live/Dead Distinction in Microbial Ecology. Appl Environ Microbiol **73**:5111-5117.

29. **Raso T., O. Bianco, B. Grosso, M. Zucca, and D. Savoia**. 2008. *Achromobacter xylosoxidans* respiratory tract infections in cystic fibrosis patients. APMIS **116**:837-841.

30. **Schwieger F., and C. C. Tebbe**. 1998. A New Approach to Utilize PCR–Single-Strand-Conformation Polymorphism for 16S rRNA Gene-Based Microbial Community Analysis. Appl Environ Microbiol. **64**:4870–4876.

31. **Steele H. L., and W. R. Streit**. 2005. Metagenomics: Advances in ecology and biotechnology. FEMS Microbiol Lett **247**:105-111.

32. **Weinbauer M. G., C. Beckmann, and M. G. Höfle**. 1998. Utility of Green Fluorescent Nucleic Acid Dyes and Aluminum Oxide Membrane Filters for Rapid Epifluorescence Enumeration of Soil and Sediment Bacteria. Appl Environ Microbiol. **64**:5000–5003.

33. **Weinbauer M. G., I. Fritz, D. F. Wenderoth, and M. G. Höfle**. 2002. Simultaneous Extraction from Bacterioplankton of Total RNA and DNA Suitable for Quantitative Structure and Function Analyses. Appl Environ Microbiol. **68**:1082–1087.

34. **Wilbanks S., and A. Glazer**. 1993. Rod structure of a phycoerythrin II-containing phycobilisome. I. Organization and sequence of the gene cluster encoding the major phycobiliprotein rod components in the genome of marine *Synechococcus* sp. WH8020. J. Biol. Chem. **268**:1226-1235.

35. **Wintzingerode F. V., U. B. Göbel, and E. Stackebrandt**. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev **21**:213-229.

36. **Yentsch C. M., P. K. Horan, K. Muirhead, Q. Dortch, E. Haugen, L. Legendre, L. S. Murphy, M. J. Perry, D. A. Phinney, S. A. Pomponi, R. W. Spinrad, M. Wood, C. S. Yentsch, and B. J. Zahuranec**. 1983. Flow Cytometry and Cell Sorting: A Technique for Analysis and Sorting of Aquatic Particles. Limnology and Oceanography **28**:1275-1280.

37. **Zwart G., B. Crump, M.P. Kamst-van-Agterveld, F. Hagen, and S. Han**. 2002. Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquat Microb Ecol. **28**:141-155.

3.8 Supplementary material

Supplementary Tab. 1. Taxonomic identification of single phylotypes found in the DNA-based SSCP fingerprints shown in Fig. 4a. Sample origin: L, only present in "live", membrane intact sorted fraction; D, only present in "dead", membrane injured sorted fraction; LD, present in both fractions.

Phylotype	Sample origin	GenBank accession no.	Taxonomic group	Closest 16S rRNA gene sequence (Accession no.)	source of closest sequence	% Similarity	Closest described species (Accession no.)	% Similarity
4	LD	GQ917124	*Betaproteobacteria*	Uncultured beta proteobacterium clone A23YP01RM (FJ569567.1)	soil, snow melt site	100	*Ralstonia syzygii* T (U28237)	100
35	LD	GQ917152	*Bacteriodetes*	Uncultured bacterium clone Lc2yS22_ML_205 (FJ355014.1)	lake Charles	99	*Sediminibacterium salmoneum* strain NJ-44 (EF407879.1)	98
37	LD	GQ917153	*Bacteriodetes*	Uncultured Bacteroidetes bacterium from DGGE gel band S1 (AY184382.1)	lake Stor Sandsjon	100	*Sediminibacterium ginsengisoli* strain DCY13 (EF067860.1)	94
39	D	GQ917154	*Bacteriodetes*	Uncultured Pedobacter sp. clone RUGL1-94 (GQ421069.1)	soil	93	*Pedobacter composti* (AB267720.1)	93
40	D	GQ917155	*Bacteriodetes*	Uncultured bacterium clone nbw601b12c1 (GQ115765.1)	skin	99	*Cloacibacterium normanense* T (AJ575430)	99
41	L	GQ917156	*Bacteriodetes*	Uncultured Bacteroidetes bacterium DGGE gel band FD 15 (DQ385020.1)	Baltic Sea water	99	*Polaribacter glomeratus* strain KOPRI_22229 (EU000227.1)	93

42	L	GQ917157	*Bacteriodetes*	Uncultured Bacteroidetes bacterium clone OU-3-1-1-L (EU626662.1)	sea urchin	98	*Lutibacter litoralis* T (AY962293)	98
43	L	GQ917158	*Bacteriodetes*	Uncultured Bacteroidetes bacterium clone NUD-17-1-1 (EU626712.1)	sea urchin	97	*Tenacibaculum mesophilum* (AB032504.1)	86
44	LD	GQ917159	*Alphaproteobacteria*	Uncultured bacterium clone LC10_L05A11 (FJ546770.1)	lake Cadagno	99	*candidatus Pelagibacter ubique/ Wolbachia pipientis* (AJ548800)	83
45	L	GQ917160	*Planctomycetes*	Uncultured bacterium clone FFCH623 (EU135171.1)	soil	93	*Gemmata obscuriglobus* (X85248)	87
46	LD	GQ917161	*Cyanobacteria*	Uncultured Synechococcus sp. clone XZNMC83 (EU703265.1)	oligosaline lake	100	*Synechococcus rubescens* SAG 3.81 (AM709629.1)	98
47	LD	GQ917162	*Cyanobacteria*	Uncultured cyanobacterium from DGGE band ESBAC-4 (AM261464.1)	lake Estanya	88	*Synechococcus rubescens* SAG 3.81 (AM709629.1)	86
48	LD	GQ917163	*Actinobacteria*	Uncultured bacterium clone metagen16S_cs_97 (FJ447619.1)	lake Bourget	99	*Iamibacter majanohamensis* (AB360448)	87
49	LD	GQ917164	*Actinobacteria*	Uncultured bacterium clone YU201C01 (FJ694627.1)	Yukon river	100	*Demequina aestuarii* (DQ010160)	91
50	L	GQ917165	*Gammaproteobacteria*	Uncultured bacterium clone FFCH895 (EU134767.1)	soil	93	*Methylobacter alcaliphilus* (EF495157)	87
51	D	GQ917166	*Gammaproteobacteria*	*Stenotrophomonas acidaminiphila* strain ST32 (FJ982935.1)	waste water sludge	100	*Stenotrophomonas acidaminiphila* strain ST32 (FJ982935.1)	100

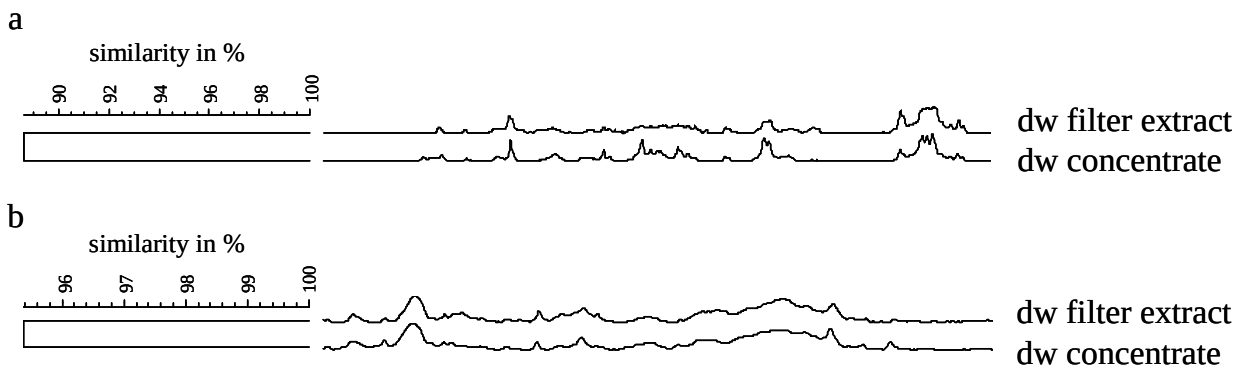
52	LD	GQ917167	*Betaproteobacteria*	Uncultured bacterium clone 081127-Aspo-Fracture-Biofilm-KA1362A06 (GQ240219.1)	groundwater biofilm	100	*Acidovorax facilis* strain 228 (EU730927.1)	99
54	LD	GQ917168	*Betaproteobacteria*	Uncultured *Bordetella sp*. clone F3feb.47 (GQ417631.1)	biological degreasing system	87	*Kerstersia gyiorum* strain LMG 5906 (NR_025669.1)	87
55	L	GQ917169	*Betaproteobacteria*	Uncultured bacterium clone 3C003283 (EU801904.1)	Chesapeake Bay	84	*Polynucleobacter necessarius* (FN429668.1)	84
56	L	GQ917170	*Betaproteobacteria*	Uncultured bacterium clone LC10_L05C06 (FJ546788.1)	lake Cadagno	98	*Polynucleobacter necessarius* (FN429668.1)	98
57	L	GQ917171	*Betaproteobacteria*	Uncultured Burkholderiaceae bacterium clone LW18m-2-18 (EU642357.1)	lake Michigan	96	*Polynucleobacter necessarius subsp. asymbioticus* (FN429668.1)	86
58	LD	GQ917172	*Betaproteobacteria*	Uncultured beta proteobacterium clone LW18m-1-70 (EU642286.1)	lake Michigan	99	*Methylophilus methylotrophus* (GQ175365)	95
61	D	GQ917173	*Betaproteobacteria*	*Polynucleobacter necessarius* strain: USHIF010 (AB470464.1)	lake Ushikunuma	99	*Polynucleobacter necessarius* (FN429657)	99
62	L	GQ917174	*Planctomycetes*	Uncultured sludge bacterium A12 (AF234727)	wastewater sludge	94	*Zavarzinella formosa* T (AM162406)	87

Supplementary Tab. 2. **Taxonomic identification of single phylotypes found in the RNA-based SSCP fingerprints shown in Fig. 4b.** N.A., not applicable (i.e., the closest described species has a similarity of < 75%). Sample origin: L, only present in “live”, membrane intact sorted fraction; D, only present in “dead”, membrane injured sorted fraction; LD, present in both fractions.

Phylotype	Sample origin	GenBank accession no.	Taxonomic group	Closest 16S rRNA gene sequence (Accession no.)	source of closest sequence	% Similarity	Closest described species (Accession no.)	% Similarity
1	LD	GQ917122	*Betaproteobacteria*	Uncultured bacterium clone 1C227656 (EU799977.1)	Newport harbour	100	*Acidovorax facilis* strain TSWCSN46 (GQ284412.1)	99
2	LD	GQ917123	*Betaproteobacteria*	Uncultured beta proteobacterium clone 500M5_F3 (DQ514229.1)	deglaciated soil	87	*Thauera terpenica* strain 21Mol (AJ005818.1)	87
4	LD	GQ917124	*Betaproteobacteria*	Uncultured *Ralstonia* sp. from DGGE gel band C4 (GQ255450.1)	shellfish hemolymph	100	*Ralstonia insidiosa* (FJ772078)	100
5	LD	GQ917125	*Betaproteobacteria*	Uncultured anaerobic bacterium clone C-147 (DQ018816.1)	anaerobic swine lagoon	83	*Thauera mechernichensis* (Y17590)	83
6	L	GQ917126	*Gammaproteobacteria*	freshwater Bacterium A2(2009) (GQ398339.1)	river biofilm	98	*Moraxella osloensis* strain FR1_63 (EU373514.1)	98
7	L	GQ917127	*Gammaproteobacteria*	Uncultured bacterium clone 2B20 (EU835445.1)	Reverse osmosis membrane biofilm	98	*Legionella erythra* T (Z32638)	96
8	L	GQ917128	*Gammaproteobacteria*	Uncultured bacterium clone 1B17 (EU835422.1)	Reverse osmosis membrane biofilm	80	*Legionella erythra* T (Z32638)	81
9	D	GQ917129	*Gammaproteobacteria*	Uncultured bacterium clone YSK16S-15 (EF612978.1)	acid mine drainage	91	*Legionella pneumophila; Alcoy 2300/99* (EU054324)	88
10	D	GQ917130	*Gammaproteobacteria*	*Pseudomonas koreensis* strain JDM-2 (GQ368179.1)	farm soil	99	*Pseudomonas koreensis* strain JDM-2 (GQ368179.1)	99
11	D	GQ917131	*Gammaproteobacteria*	Uncultured bacterium clone nbw232g03c1 (GQ069759.1)	skin	98	*Pseudomonas putida* strain GNL8 (FJ768454.1)	98
12	L	GQ917132	*Gammaproteobacteria*	Uncultured bacterium from SSCP band RNA 2-8-7 (DQ077602.1)	drinking water supply system	100	*Methylocaldum gracile* (U89298)	92
13	D	GQ917133	*Nitrospira*	Uncultured bacterium clone 3BR-3AA (EU937879.1)	freshwater biofilm	88	*Nitrospira moscoviensis* T (X82558)	86
14	LD	GQ917134	*Alphaproteobacteria*	Uncultured bacterium clone W_0307_65 (GQ379456.1)	soil	97	*Bosea vestrisii* T (AF288306)	97

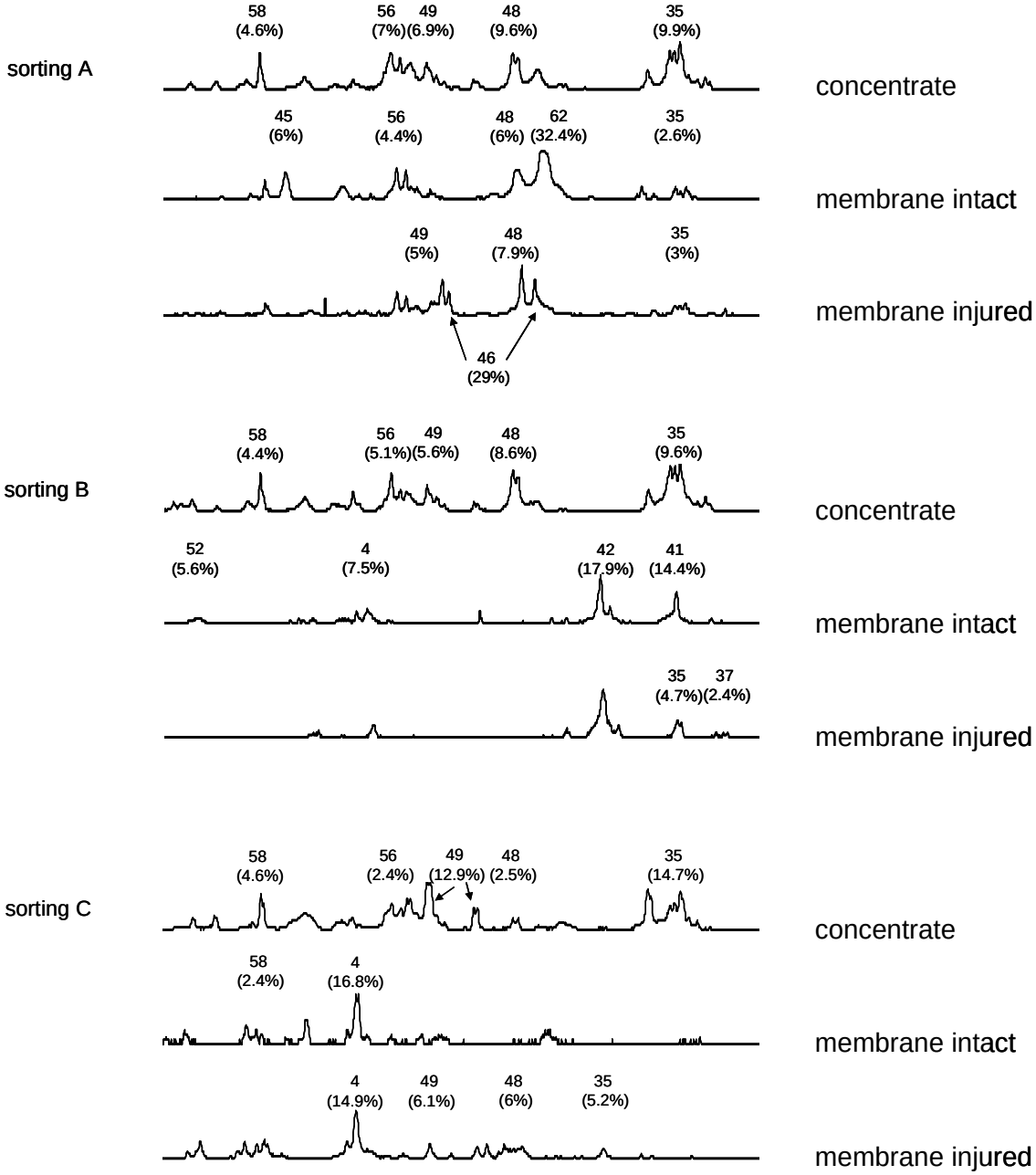
15	LD	GQ917135	*Alphaproteobacteria*	Uncultured alpha proteobacterium clone sw-xj62 (GQ302527.1)	cold spring	97	*Pedomicrobium americanum* (X97692)	94
16	LD	GQ917136	*Alphaproteobacteria*	Uncultured alpha proteobacterium clone GASP-KB3S3_H06 (EU298674.1)	soil	99	N.A.	
17	LD	GQ917137	*Alphaproteobacteria*	Uncultured bacterium clone 0MHA_A12 (GQ306092.1)	periglacial soil	93	*Belnapia moabensis* (AJ871428)	93
18	LD	GQ917138	*Alphaproteobacteria*	Uncultured bacterium clone P1O-78 (EU375422.1)	lake Puma Yumco	98	*Roseococcus suduntuyensis* (EU012448)	96
19	LD	GQ917139	*Gammaproteobacteria*	Uncultured bacterium clone 5C231590 (EU803928.1)	lake Gatun	96	*Methylonatrum kenyense* (EU006088)	85
20	L	GQ917140	*Firmicutes*	Uncultured bacterium clone KO2_aai19h11 (EU776338.1)	Kangaroo feces	81	*Ruminococcus flavefaciens* strain AR72 (AF104841.1)	81
21	LD	GQ917141	*Planctomycetes*	Uncultured planctomycete, clone DSP41 (AJ290189.1)	river Spittelwasser biofilm	94	*Rhodopirellula baltica* (FJ624344)	85
22	L	GQ917142	*Bacteriodetes*	Uncultured bacterium clone HH1409 (FJ502249.1)	lake Cadagno	98	*Pedobacter sp.* Tianshan 221-3 (EU305635.1)	93
23	D	GQ917143	*Bacteriodetes*	Uncultured Bacteroidetes bacterium clone A21YG08RM (FJ568900.1)	soil at snow melt site	95	*Flexibacter canadensis* (AB078046)	89
24	LD	GQ917144	*Chloroflexi*	Uncultured bacterium clone 538.F4 (EU357588.1)	soil	99	N.A.	
25	LD	GQ917145	*Chloroflexi*	Uncultured bacterium clone: CMBR-4 (AB305032.1)	wastewater treatment plant	91	*Caldilinea aerophila* (AB067647)	83
26	L	GQ917146	*Cyanobacteria*	Uncultured bacterium from SSCP band Li-8R-10-2 (DQ077567.1)	drinking water supply system	95	*Glaucocystis nostochinearum* (X82496)	79
27	L	GQ917147	*Cyanobacteria*	Uncultured bacterium from SSCP band TW15-RNA1-14-2 (DQ077556.1)	drinking water supply system	94	*Glaucocystis wittrockiana* (X82495)	83
28	LD	GQ917148	*Bacteriodetes*	Uncultured bacterium clone F126 (FJ348594.1)	waste water sludge	99	*Thermolithobacter carboxydivorans* (DQ095862)	90

29	D	GQ917149	*Cyanobacteria*	Uncultured bacterium clone IFBC1H11 (EU592534.1)	freshwater lake	88	*Synechococcus sp. KORDI-78* (FJ497748.1)	87
31	LD	GQ917150	*Cyanobacteria*	Uncultured bacterium clone N05Dec-74 (EU442941.1)	lake Nam Co	90	*Cyanobium sp.* JJM10A4 (AM710358.1)	90
32	L	GQ917151	*Cyanobacteria*	Uncultured bacterium clone LaP15L91 (EF667687.1)	river sediment	97	*Synechococcus sp.* MH305 (AY224198.1)	100
46	LD	GQ917161	*Cyanobacteria*	Uncultured Synechococcus sp. clone XZNMC83 (EU703265.1)	lake Namucuo	100	*Synechococcus rubescens* SAG 3.81 (AM709629.1)	98



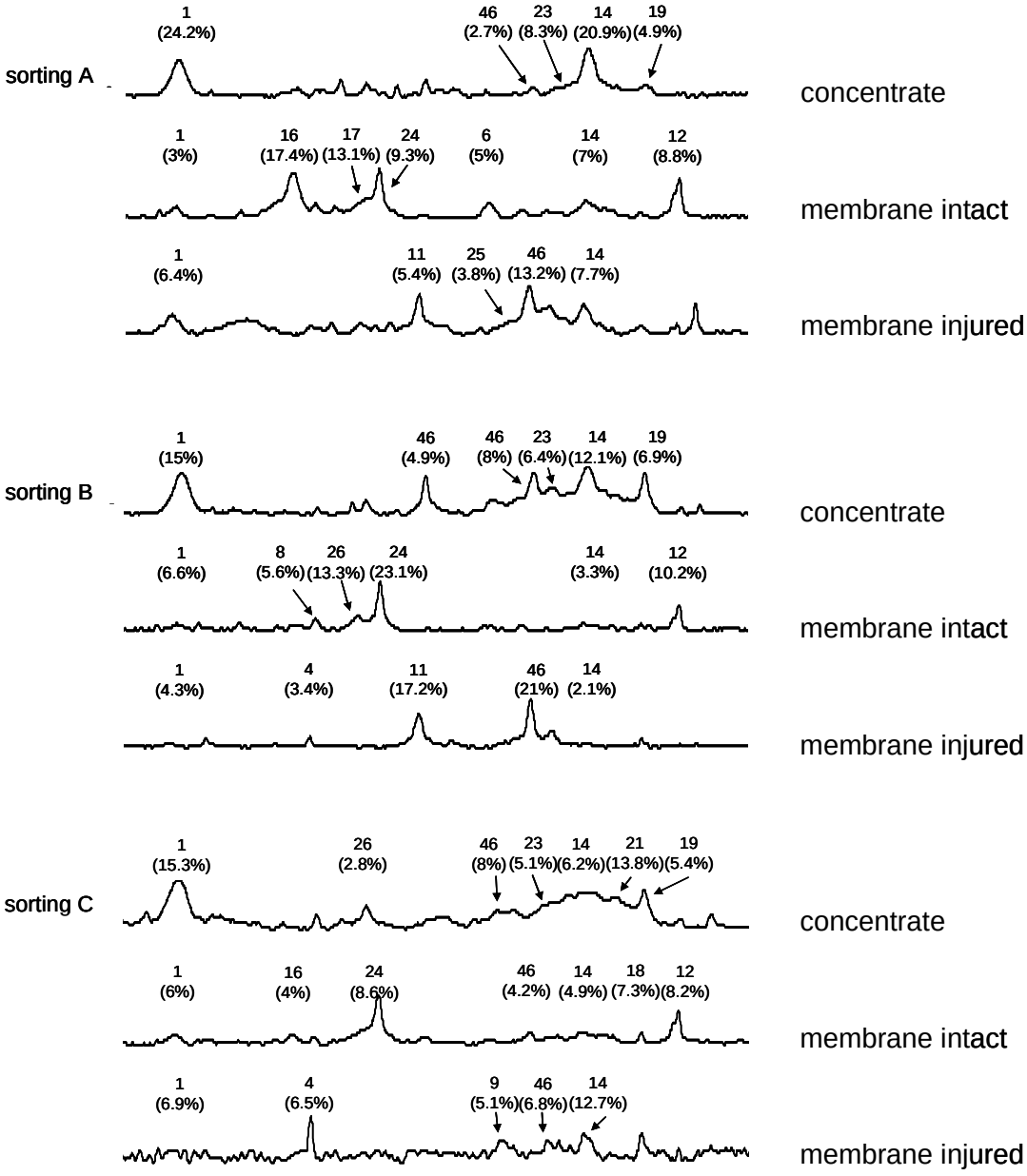
Supplementary Fig 1. Comparison of SSCP electropherograms of concentrated drinking water samples directly extracted from concentrates and non-concentrated drinking water extracted from filter sandwiches using GelCompare II. (a) DNA-based SSCP electropherograms (b) RNA-based SSCP electropherograms.

2a)



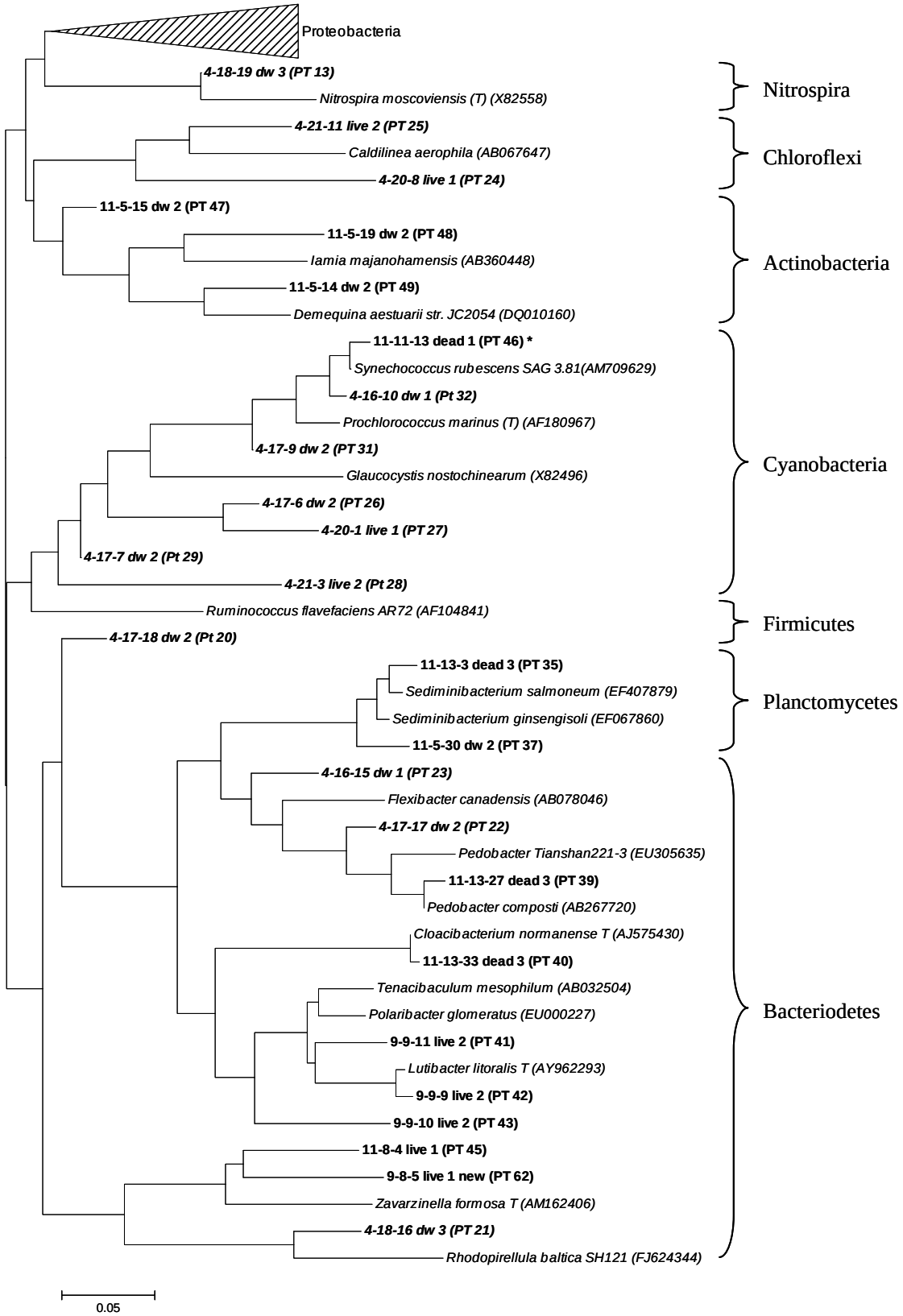
Supplementary Fig. 2. **(a)** Detailed analysis of the electropherograms from the different sampling dates originating from the DNA-based SSCP gel given in Fig. 4a. Numbers correspond to the phylotypes given in Tab 1. Percentages in parentheses represent relative abundances of phylotypes. **(b)** Detailed analysis of the electropherograms from the different sampling dates originating from the RNA-based SSCP gel given in Fig. 4b.

2 b)

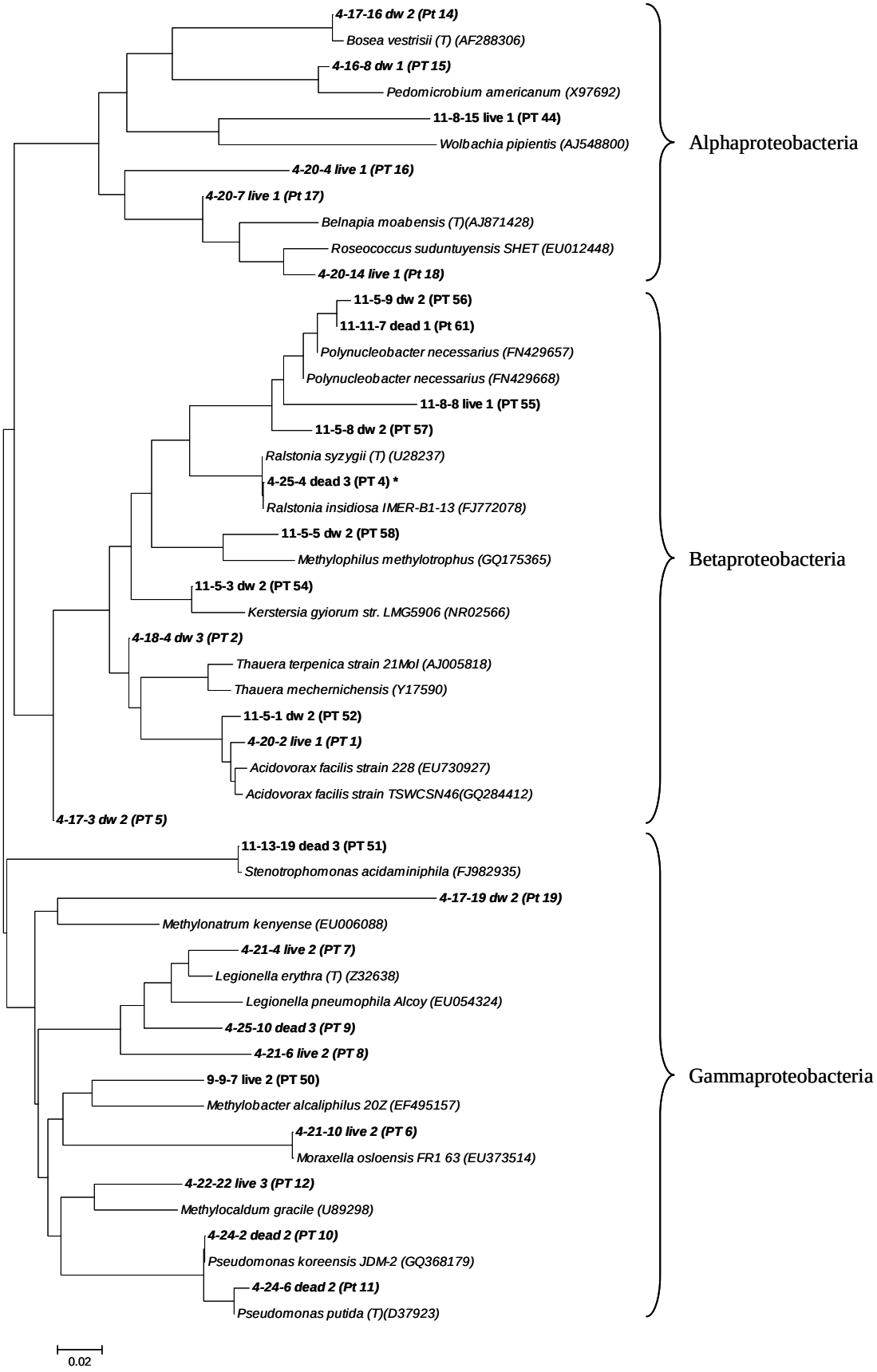


Supplementary Fig 3. **Phylogenetic analysis of 16S rRNA gene sequences obtained from the bands of the SSCP fingerprints shown in Fig. 4 using the neighbor-Joining method.** The optimal tree with the sum of branch length = 2.1515 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pair wise sequence comparisons (pair wise deletion option). Sequences are labeled with their origin plus the phylotype number (in parenthesizes) given in supplementary Table 1 and 2. Sequences are coded with different character types according to their origin in terms of nucleic acid type: DNA-based sequences are shown in bold, RNA-based sequences are shown in bold italic, and sequences occurring in DNA- and RNA-based fingerprints are shown in bold with an asterisk. (a) Phylogenetic tree of all detected phyla. (b) Detailed phylogenetic tree of the detected *Proteobacteria*.

3 a)


Proteobacteria
4-18-19 dw 3 (PT 13)
Nitrospira moscoviensis (T) (X82558)
Nitrospira
4-21-11 live 2 (PT 25)
Caldilinea aerophila (AB067647)
4-20-8 live 1 (PT 24)
Chloroflexi
11-5-15 dw 2 (PT 47)
11-5-19 dw 2 (PT 48)
Iamia majanohamensis (AB360448)
11-5-14 dw 2 (PT 49)
Demequina aestuarii str. JC2054 (DQ010160)
Actinobacteria
11-11-13 dead 1 (PT 46) *
Synechococcus rubescens SAG 3.81(AM709629)
4-16-10 dw 1 (Pt 32)
Prochlorococcus marinus (T) (AF180967)
4-17-9 dw 2 (PT 31)
Glaucocystis nostochinearum (X82496)
4-17-6 dw 2 (PT 26)
4-20-1 live 1 (PT 27)
4-17-7 dw 2 (Pt 29)
4-21-3 live 2 (Pt 28)
Cyanobacteria
Ruminococcus flavefaciens AR72 (AF104841)
Firmicutes
4-17-18 dw 2 (Pt 20)
11-13-3 dead 3 (PT 35)
Sediminibacterium salmoneum (EF407879)
Sediminibacterium ginsengisoli (EF067860)
11-5-30 dw 2 (PT 37)
Planctomycetes
4-16-15 dw 1 (PT 23)
Flexibacter canadensis (AB078046)
4-17-17 dw 2 (PT 22)
Pedobacter Tianshan221-3 (EU305635)
11-13-27 dead 3 (PT 39)
Pedobacter composti (AB267720)
Cloacibacterium normanense T (AJ575430)
11-13-33 dead 3 (PT 40)
Tenacibaculum mesophilum (AB032504)
Polaribacter glomeratus (EU000227)
9-9-11 live 2 (PT 41)
Lutibacter litoralis T (AY962293)
9-9-9 live 2 (PT 42)
9-9-10 live 2 (PT 43)
11-8-4 live 1 (PT 45)
9-8-5 live 1 new (PT 62)
Zavarzinella formosa T (AM162406)
4-18-16 dw 3 (PT 21)
Rhodopirellula baltica SH121 (FJ624344)
Bacteriodetes
0.05

3 b)


4-17-16 dw 2 (Pt 14)
Bosea vestrisii (T) (AF288306)
4-16-8 dw 1 (PT 15)
Pedomicrobium americanum (X97692)
11-8-15 live 1 (PT 44)
Wolbachia pipientis (AJ548800)
4-20-4 live 1 (PT 16)
4-20-7 live 1 (Pt 17)
Belnapia moabensis (T)(AJ871428)
Roseococcus suduntuyensis SHET (EU012448)
4-20-14 live 1 (Pt 18)
Alphaproteobacteria
11-5-9 dw 2 (PT 56)
11-11-7 dead 1 (Pt 61)
Polynucleobacter necessarius (FN429657)
Polynucleobacter necessarius (FN429668)
11-8-8 live 1 (PT 55)
11-5-8 dw 2 (PT 57)
Ralstonia syzygii (T) (U28237)
4-25-4 dead 3 (PT 4) *
Ralstonia insidiosa IMER-B1-13 (FJ772078)
11-5-5 dw 2 (PT 58)
Methylophilus methylotrophus (GQ175365)
11-5-3 dw 2 (PT 54)
Kerstersia gyiorum str. LMG5906 (NR02566)
4-18-4 dw 3 (PT 2)
Thauera terpenica strain 21Mol (AJ005818)
Thauera mechernichensis (Y17590)
11-5-1 dw 2 (PT 52)
4-20-2 live 1 (PT 1)
Acidovorax facilis strain 228 (EU730927)
Acidovorax facilis strain TSWCSN46(GQ284412)
4-17-3 dw 2 (PT 5)
Betaproteobacteria
11-13-19 dead 3 (PT 51)
Stenotrophomonas acidaminiphila (FJ982935)
4-17-19 dw 2 (Pt 19)
Methylonatrum kenyense (EU006088)
4-21-4 live 2 (PT 7)
Legionella erythra (T) (Z32638)
Legionella pneumophila Alcoy (EU054324)
4-25-10 dead 3 (PT 9)
4-21-6 live 2 (PT 8)
9-9-7 live 2 (PT 50)
Methylobacter alcaliphilus 20Z (EF495157)
4-21-10 live 2 (PT 6)
Moraxella osloensis FR1 63 (EU373514)
4-22-22 live 3 (PT 12)
Methylocaldum gracile (U89298)
4-24-2 dead 2 (PT 10)
Pseudomonas koreensis JDM-2 (GQ368179)
4-24-6 dead 2 (Pt 11)
Pseudomonas putida (T)(D37923)
Gammaproteobacteria
0.02

CHAPTER 4

Polyvalent Fingerprint Based Molecular Surveillance Methods for Drinking Water Supply Systems

Karsten Henne, Leila Kahlisch, Josefin Draheim, Manfred G. Höfle and Ingrid Brettar

Dept. Vaccinology and Applied Microbiology, Helmholtz Center for Infection Research (HZI), Inhoffenstrasse 7, 38124 Braunschweig, Germany

CHAPTER 4 Polyvalent Fingerprint Based Molecular Surveillance Methods for Drinking Water Supply Systems

4.1 Abstract

Despite the relevance for public health, surveillance of drinking water supply systems (DWSS) in Europe is mainly achieved by cultivation based detection of indicator bacteria. The study presented here demonstrates the use of molecular analysis based on fingerprints of DNA extracted from drinking water bacteria as a valuable monitoring tool of DWSS and was exemplified for a DWWS in Northern Germany. The analysis of the bacterial community of drinking water was performed by a set of 16S rRNA gene based fingerprints, sequence analysis of relevant bands and phylogenetic assignment of the 16S rRNA sequences. We assessed the microflora of drinking water originating from two reservoirs in the Harz Mountains. The taxonomic composition of the bacterial communities from both reservoirs was very different at the species level reflecting the different limnological conditions. Detailed analysis of the seasonal community dynamics of the tap water revealed a significant influence of both source waters on the composition of the microflora and demonstrated the relevance of the raw water microflora for the drinking water reaching the consumer. According to our experience, molecular analysis based on fingerprints of different degrees of resolution can be considered as a valuable monitoring tool of DWSS.

4.2 Introduction

Despite the relevance for public health, surveillance of drinking water supply systems in Europe is mainly achieved by cultivation based detection of indicator bacteria. This approach bears the risk of neglecting viable but nonculturable (VNBC) bacteria on the one hand, on the other hand, many pathogenic bacteria, including emerging ones are not monitored (8, 15, 21). Careful estimates indicate that each year about 350 million people are infected by waterborne pathogens with 10-20 millions succumbing to severe cases (WHO (23)). This phenomenon is far from being restricted to developing countries but also threatens developed countries. In the USA almost 430,000 cases were reported in 126 outbreaks of waterborne infectious diseases from 1991 to 1999 (1).

Production of drinking water complying with international quality standards does not necessarily ensure good drinking water for the consumer (2). Re-growth of bacteria in the distribution system is a major problem that may have adverse effects on drinking water quality and is correlated with biofilm formation. The effects of re-growth may range from effects on taste and odour to true health threats when it comes to re-growth of pathogenic bacteria (20). Key factors influencing re-growth of bacteria in a drinking water supply system (DWSS) are: i) concentration of organic compounds, ii) chlorine concentration, iii) residence

time of the water in the distribution system, iv) water temperature and v) physico-chemical characteristics of the material lining the distribution pipes (13).

The bacterial community of drinking water plays a crucial role for the drinking water quality. It is the main consumer of the organic carbon in the drinking water, mineralizes it to CO_2 or other degradation products, nitrifies ammonium to nitrite and nitrate, and forms biofilms. The autochthonous microflora can sustain the growth of protozoa and metazoa (e.g. crustacean) that are visible to the consumer (4, 19) or may have adverse effects on the taste and safety of the drinking water (11). The microbial community of the drinking water may directly interfere with pathogenic bacteria, i.e. it can suppress or promote the survival and growth of hygienically relevant and potentially pathogenic bacteria (10). E.g. the formation of biofilms enables survival or even growth of pathogenic bacteria, while the competition for the same carbon sources or the production of antibiotic substances may suppress pathogenic bacteria. Since the microbial community is a key factor of drinking water quality with respect to many aspects, its analysis is a focus of our study.

The HEALTHY-WATER project, a project in the 6th Framework of the EU (http://www.hzi-helmholtz.de/en/healthy_water/) is aiming towards the development of new molecular detection technologies of microbial pathogens in drinking water with special emphasis on emerging pathogens (14). Among several approaches that are under development, fingerprint based methods and their results will be presented here, those especially have the potential to monitor the whole bacterial community and thus bear the potential to detect also unexpected pathogenic bacteria.

4.3 Methods

4.3.1 Study site

The overall study comprises samples from a DWSS in Northern Germany that provides about 80 Mio m3 of drinking water per year and is providing drinking water for about two million people. Source water of the DWSS are provided by two surface water reservoirs, an oligotrophic reservoir (Grane, pH 7.2) and a dystrophic reservoir (Ecker, pH 5.2). The collection of aerobic raw water is done from the deep water (50-58 m). More details on the DWWS are given by Eichler et al. 2006. The focus of this study is on tap water and the seasonal changes studied from autumn 2006 to spring 2008.

4.3.2 Molecular methodology

The bacterial community of the water were harvested by filtering several liters of water onto a sandwich of a glass fiber GF/F plus 0.2 µm nuclepore filter (Whatman) (for details on the molecular methods see Eichler et al. (3)). In brief: DNA was extracted and purified; bacterial 16S rRNA gene amplicons generated by PCR were subjected to

separation by non-denaturing acrylamide gel electrophoresis enabling Single Strand Conformational Polymorphism (SSCP) analysis. DNA based SSCP analyses were performed to follow the seasonal dynamics (5, 6, 18). The banding patterns on the SSCP gels, used as a direct measure of the community structure, were compared by cluster analysis (GelCompare II, Applied Maths). The composition of the bacterial community was determined by sequencing the single bands of the gel pattern and identifying the sequences by phylogenetic analysis using the international 16S rRNA gene sequence data base.

4.4 Results and Discussion

4.4.1 Overall community structure of the drinking water microflora

The overall community structure of the drinking water microflora of tap water was assessed during one and a half years at monthly intervals to understand seasonal dynamics (Figure 1). These DNA based community fingerprints are banding patterns of single 16S rRNA genes separated according to sequence differences using SSCP electrophoresis. Ideally, the single bands represent different bacterial taxa at about the species level (17). For a detailed analysis of the single banding patterns density curves were produced using an electronic scanner (Figure 2). These density patterns show peaks, corresponding to the specific bands, and allow quantification of the amount of single strand DNA present in the single bands by integrating the area under the specific peak. A first comparison of the fingerprints shows that there are three major bands (1-3 marked with arrows in Figure 1) that occurred in all samples whereas several bands occurred only during certain times of the year (boxes 4-10 in Figure 1). The banding patterns of the single drinking water communities comprise about 40 to 80 different bands above the relative abundance threshold of 0.1% of the total DNA per lane. The constant bands represent 6-24% of the total DNA per lane leaving about 59-87% of the DNA for the variable bands. A seasonal pattern of the three constant bands can be recognized by comparing their relative amounts (Figure 3). Especially the most abundant band 3 shows a strong increase, from 3.6 to 16%, in October and a decline in January to March in both winters studied. Overall, these constant bands can be assumed to represent three different bacterial species that showed seasonal changes in their relative abundances by a factor of four according to the DNA abundance of the band. For a detailed understanding of the variation in the banding patterns, i. e. the community structure of the whole bacterial drinking water microflora, a cluster analysis was performed that allows a statistical comparison of the banding patterns of the different lanes (Figure 4). The cluster analysis revealed that the banding patterns changed in about 3 to 4 month intervals as revealed by the six main clusters in Figure 4. In addition, the cluster late summer 07 forms a subgroup with cluster autumn 07 as well as cluster winter 08 with spring 08. This sub-

grouping indicates that the bacterial microflora is continuously changing, but mostly still related to the previous microflora.

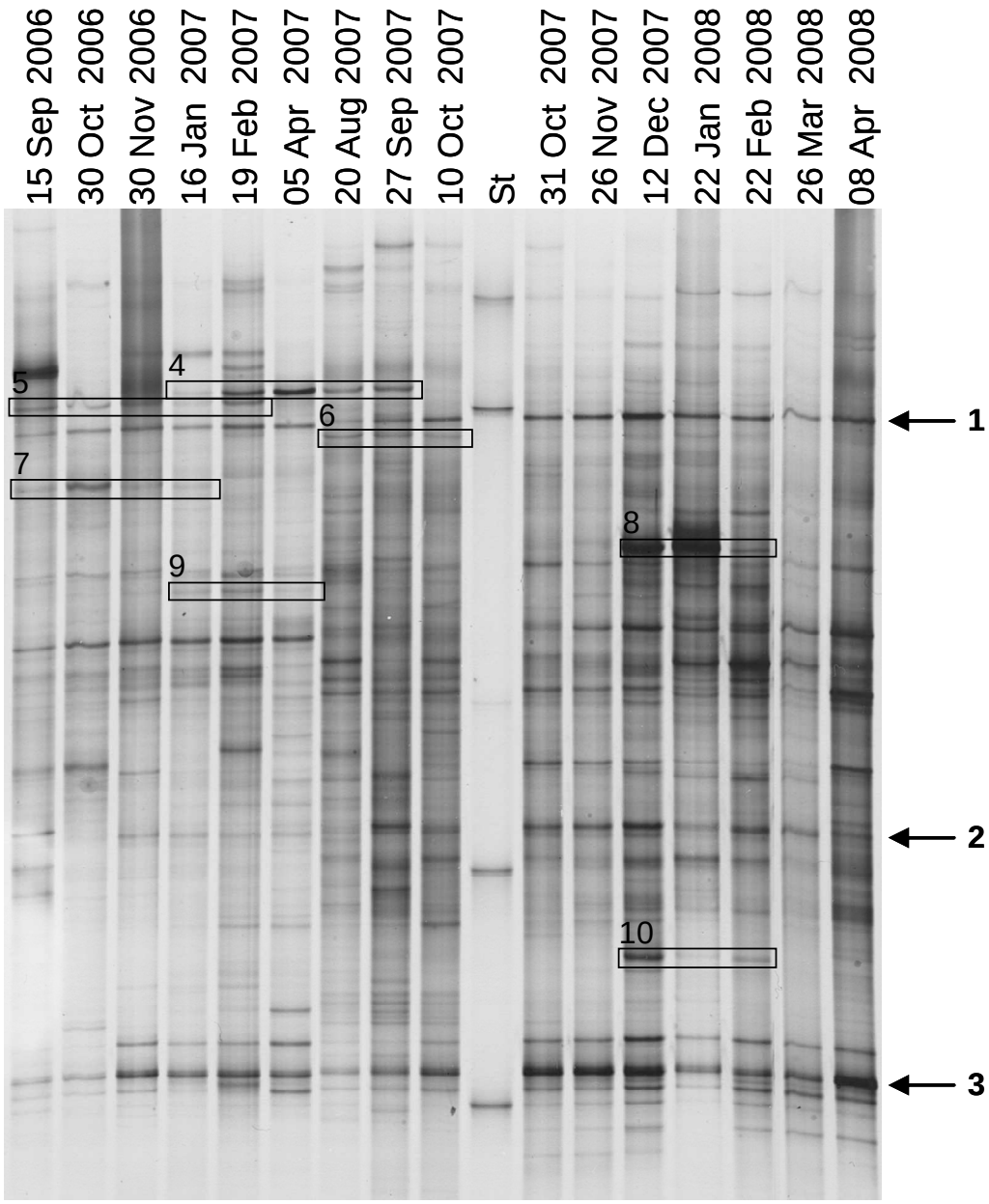


Fig. 1. DNA based community fingerprints from tap water samples obtained at the indicated dates. Arrows indicate bands observed in all samples, bands in boxes are only observed during certain times of the year. St= standards of reference bacterial species.

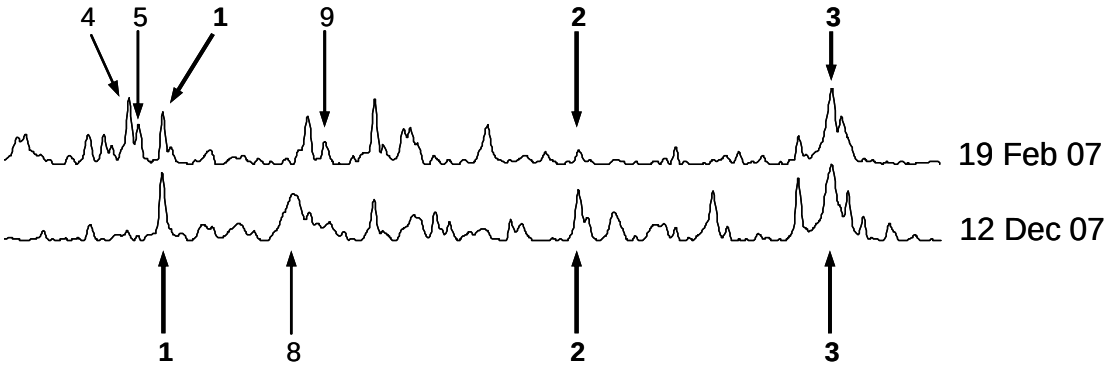


Fig. 2. Density curves from the banding pattern of the community fingerprints from two different samples (constant bands 1-3 in bold). Band numbering is consistent with Figure 1.

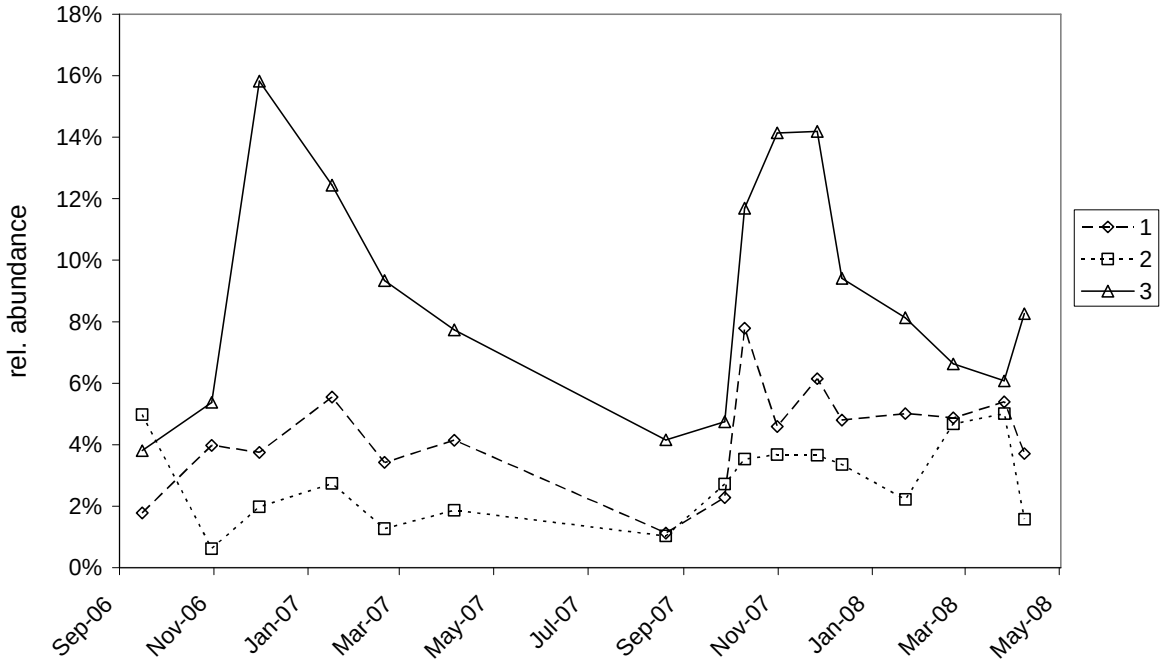


Fig. 3. Seasonal variation of the relative abundances of the single strand DNA of the three major bands (1-3) representing three different bacterial species.

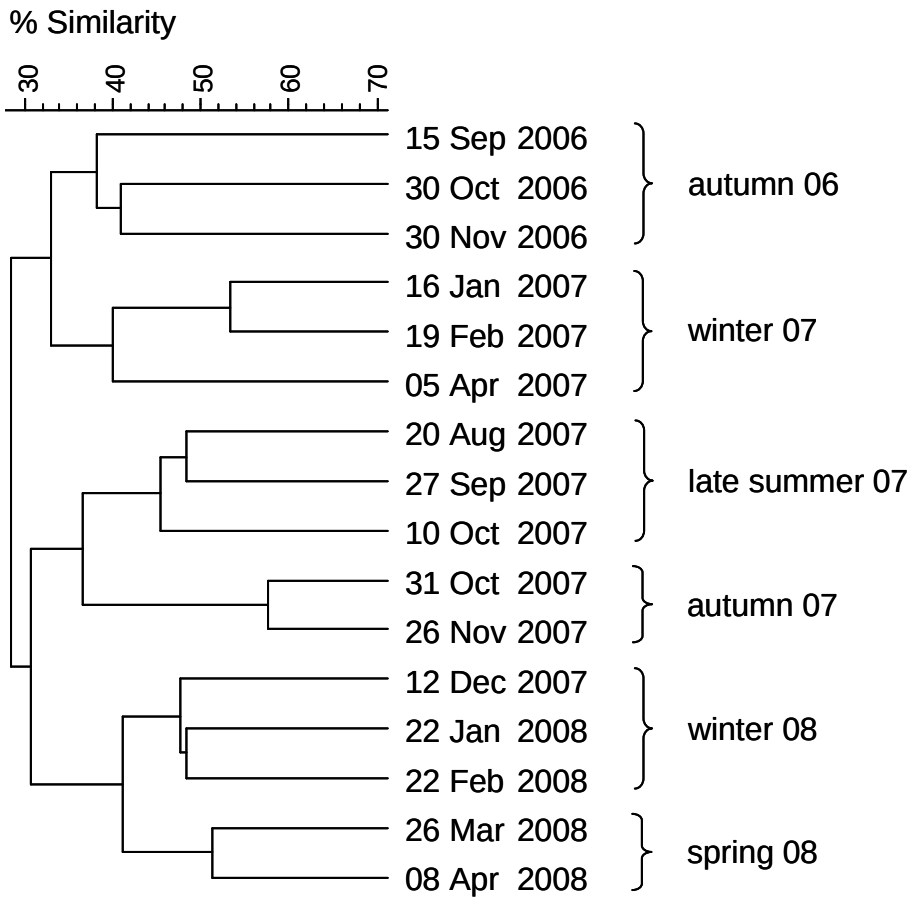


Fig. 4. Cluster analysis of all banding patterns from the community fingerprints shown in Figure 1 (analysis was done by using GelCompare II (Applied Maths), Algorithms: Dice, Complete Linkage, all bands above 0.1% abundance included in analysis).

4.4.2 Taxonomic composition of the drinking water microflora

For identification of the single bacterial taxa represented by the bands of the community fingerprints, these bands have to be excised and sequenced. The generated 16S rRNA partial sequences (about 420nt) can then be compared with the large data set of bacterial 16S rRNA sequences available in international databases to identify the closest known bacterial species. In a previous study of the same DWSS, we identified 71 unique phylotypes, i. e. 16S rRNA gene sequences with a sequence similarity of > 98% and phylogenetic uniqueness as discrimination criteria that comprised most of the bacterial species in this drinking water community (3). Using these phylotypes as a reference data base, we could identify the three constant bands as the following bacterial taxa: band 1 = *Methylophilus* sp. (identical to phylotype 1 from Eichler et al. (3), class *Betaproteobacteria*); band 2 = identical to phylotype 21 from Eichler et al. (3), phylum *Actinobacteria*); band 3 = identical to phylotype 22 from Eichler et al. (3), class *Alphaproteobacteria*). All three phylotypes belonged to bacterial species that have not been cultured and could only be

identified by molecular analysis of DNA extracted from drinking water. In addition, all three phylotypes belonged to different bacterial classes or phyla indicating a large phylogenetic diversity of the drinking water microflora (22). As pointed out above, several bands (number 4 to 10) occurred only during a specific period and can be seen as indicators of changes in the structure and composition of the drinking water microflora. Sequence comparison of band 8 revealed that it was identical with phylotype 6 from Eichler et al. (3) representing a betaproteobacterium from the genus *Simonsiella*. This phylotype had only been observed before in the dystrophic Eker reservoir microflora and can therefore be considered as an indicator for this microflora.

The analysis of the bacterial community by SSCP fingerprints has already been shown to be of great use for the study of the impact of the source water and the water treatment processes on the drinking water bacterial community. Eichler et al. (3) have shown that the bacterial community structure of the raw water samples from the two reservoirs was very different reflecting the different limnological conditions of the reservoirs (highly dystrophic versus oligotrophic reservoir). No major changes of the structure of the bacterial community were observed after flocculation and sand filtration, while chlorination of the processed raw water strongly affected bacterial community structure as best reflected by the RNA-based fingerprints. According to assessment of the community composition by sequencing of abundant bands and phylogenetic analysis of the sequences obtained, the taxonomic composition of the bacterial communities from both reservoirs was very different. After chlorination, growth of nitrifying bacteria was observed. Detailed analysis of the community dynamics of the whole DWSS revealed a significant influence of both source waters on the composition of the microflora and demonstrated the relevance of the raw water microflora for the drinking water provided to the end user.

4.5 Conclusions

- The DNA based community fingerprints allowed to follow the seasonal dynamics of the whole bacterial microflora in tap water.

- The SSCP fingerprints enabled the assessment of the relative abundance of all bacterial members of the drinking water microflora to a threshold of 0.1% relative abundance and, after sequencing, their taxonomic identification to the species level.

- The seasonal dynamics of the tap water microflora was characterized by three constant and 40-80 varying members of the bacterial community.

These insights into the bacterial community dynamics of a drinking water supply system obtained during this and the former study led us to recommend molecular analysis based on fingerprints of different degrees of resolution as a valuable monitoring tool of DWSS. The rapid overview gained on the DWSS bacterial community can be furthermore improved and accelerated by standardized formats of the molecular analysis.

4.5.1 Future perspectives and applications of fingerprints as tools for drinking water research and monitoring

In the future, SSCP analysis can be used to focus on specific pathogenic bacterial groups of interest what is currently under development in the Healthy-Water project. To achieve this goal, primers with a different degree of specificity are designed and applied to generate fingerprints for pathogenic bacterial genera or species of interest such as *Campylobacter, Arcobacter* and *Helicobacter* (12, 16). Especially, with respect to biofilms, analysis of DWSS for these genera are of high relevance to human health (9, 21).

In many cases a higher phylogenetic resolution is needed than the one retrievable from the fingerprint band sequences in order to get a more precise taxonomic position of the target pathogenic bacterium. An improvement of the phylogenetic resolution can be achieved by designing highly specific primers and probes of a different degree of specificity based on the sequence of bands of interest (7). Using these highly specific primers allows the generation of a complete 16S rRNA gene sequence (>1400 nucleotides) of aquatic bacteria (7). This full 16S rRNA sequence allows a more precise analysis of the phylogenetic affiliation compared to the fragments obtained from the SSCP gel (about 420 nucleotides). Additionally, quantification of specific (pathogenic) bacteria by real-time PCR can be linked to SSCP-fingerprints. The above mentioned primers designed based on the fingerprint band sequences can be used for real-time PCR. This is of specific relevance when a new organism is detected by fingerprints that are of interest, e.g. suspicious to be a pathogenic or noxious bacterium, but not yet cultivated and the 16S rRNA sequence is not yet available in public data bases. These examples demonstrate the great potential of molecular fingerprint analyses for an improved monitoring of DWSS and a better understanding of possible hygienic risks related to various treatment and management procedures.

4.6 Acknowledgements

The technical assistance by Julia Strömpl is greatly acknowledged. This work was supported by funds from the European Commission for the HEALTHY WATER project (FOOD-CT-2006-036306). The authors are solely responsible for the content of this publication. It does not represent the opinion of the European Commission. The European Commission is not responsible for any use that might be made of data appearing therein.

4.7 References

1. **Craun G. F., N. Nwachuku, R. L. Calderon, and M. F. Craun**. 2002. Outbreaks in Drinking-Water Systems, 1991-1998. J Environ Health **65**:16–23.

2. **Dewettinck T., E. Van Houtte, D. Geenens, K. Van Hege, and W. Verstraete**. 2001. HACCP (Hazard Analysis and Critical Control Points) to guarantee safe water reuse and drinking water production- A case study. Water Science & Technology **43**:31–38.

3. **Eichler S., R. Christen, C. Höltje, P. Westphal, J. Bötel, I. Brettar, A. Mehling, and M. G. Höfle**. 2006. Composition and Dynamics of Bacterial Communities of a Drinking Water Supply System as Assessed by RNA- and DNA-Based 16S rRNA Gene Fingerprinting. Appl. Environ. Microbiol. **72**:1858–1872.

4. **Gauthier V., B. Gérard, J. Portal, J. Block, and D. Gatel**. 1999. Organic matter as loose deposits in a drinking water distribution system. Water Res **33**:1014-1026.

5. **Hammes F., E. Salhi, O. K□ster, H. P. Kaiser, T. Egli, and U. von Gunten**. 2006. Mechanistic and kinetic evaluation of organic disinfection by-product and assimilable organic carbon (AOC) formation during the ozonation of drinking water. Water Res **40**:2275-2286.

6. **Hoefel D., P. Monis, W. Grooby, S. Andrews, and C. Saint**. 2005. Culture-independent techniques for rapid detection of bacteria associated with loss of chloramine residual in a drinking water system. Appl Environ Microbiol **71**:6479-6488.

7. **Höfle M. G., S. Flavier, R. Christen, J. Botel, M. Labrenz, and I. Brettar**. 2005. Retrieval of nearly complete 16 S rRNA gene sequences from environmental DNA following 16 S rRNA-based community fingerprinting. Environ Microbiol **7**:670–675.

8. **Huq A., I. N. Rivera, and R. R. Colwell**. 2000. Epidemiological significance of viable but nonculturable microorganisms, S. 301–323. *In* Nonculturable Microorganisms in the Environment.

9. **Juhna T., D. Birzniece, S. Larsson, D. Zulenkovs, A. Sharipo, N. F. Azevedo, F. Menard-Szczebara, S. Castagnet, C. Feliers, and C. W. Keevil**. 2007. Detection of Escherichia coli in Biofilms from Pipe Samples and Coupons in Drinking Water Distribution Networks. Appl. Environ. Microbiol. **73**:7456-7464.

10. **LeChevallier M. W.** 1990. Coliform regrowth in drinking water: a review. J Am Water Works Assoc **82**:74–86.

11. **Mallevialle J., and I. H. Suffet**. 1981. Identification and treatment of tastes and odors in drinking water. AWWA 299.

12. **Moreno Y., J. L. Alonso, S. Botella, M. A. Ferrús, and J. Hernández**. 2004. Survival and injury of Arcobacter after artificial inoculation into drinking water. Res Microbiol **155**:726-730.

13. **Niquette P., P. Servais, and R. Savoir**. 2001. Bacterial Dynamics in the drinking water distribution system of Brussels. Water Res **35**:675-682.

14. **Nwachcuku N., and C. P. Gerba**. 2004. Emerging waterborne pathogens: can we kill them all? Curr Opin Biotechnol **15**:175-180.

15. **Organization for Economic Cooperation and Development (OECD)**. 2003. Safer drinking water: Improving the assessment of microbial safety. Paris, France.

16. **Sandberg M., K. Nyg\aard, H. Meldal, P. S. Valle, H. Kruse, and E. Skjerve**. 2006. Incidence trend and risk factors for *Campylobacter* infections in humans in Norway. BMC Public Health **6**:179.

17. **Schmalenberger A., F. Schwieger, and C. C. Tebbe**. 2001. Effect of Primers Hybridizing to Different Evolutionarily Conserved Regions of the Small-Subunit rRNA Gene in PCR-Based Microbial Community Analyses and Genetic Profiling. Appl. Environ. Microbiol. **67**:3557-3563.

18. **Schwieger F., and C. C. Tebbe**. 1998. A New Approach To Utilize PCR–Single-Strand-Conformation Polymorphism for 16S rRNA Gene-Based Microbial Community Analysis. Appl Environ Microbiol. **64**:4870–4876.

19. **Servais P., P. Laurent, and G. Randon**. 1995. Comparison of the bacterial dynamics in various French distribution systems. AQUA **44**:10–17.

20. **Vital M., H. P. Fuchslin, F. Hammes, and T. Egli**. 2007. Growth of *Vibrio cholerae* O1 Ogawa Eltor in freshwater. Microbiology **153**:1993-2001.

21. **Watson C., R. Owen, B. Said, S. Lai, J. Lee, S. Surman-Lee, and G. Nichols**. 2004. Detection of *Helicobacter pylori* by PCR but not culture in water and biofilm samples from drinking water distribution systems in England. J Appl Microbiol **97**:690-698.

22. **Williams M. M., J. W. S. Domingo, M. C. Meckes, C. A. Kelty, and H. S. Rochon**. 2004. Phylogenetic diversity of drinking water bacteria in a distribution system simulator. J Appl Microbiol **96**:954–964.

23. **World Health Organization (WHO)**. 1997. Division of emerging and communicable diseases surveillance and control annual report - 1996. Geneva.

CHAPTER 5

High-resolution *in situ* genotyping of Legionella pneumophila populations in drinking water by Multiple-Locus Variable-Number of Tandem Repeat Analysis (MLVA) using environmental DNA

Leila Kahlisch, Karsten Henne, Josefin Draheim, Manfred G. Höfle and Ingrid Brettar

Dept. Vaccinology and Applied Microbiology, Helmholtz Center for Infection Research (HZI), Inhoffenstrasse 7, 38124 Braunschweig, Germany

CHAPTER 5 High-resolution *in situ* genotyping of Legionella pneumophila populations in drinking water by Multiple-Locus Variable-Number of Tandem Repeat Analysis (MLVA) using environmental DNA

5.1 Abstract

Central to the understanding of infections by *Legionella pneumophila* is the detection of this waterborne pathogen at a clonal level. Currently, Multiple-Locus VNTR Analysis (MLVA) of *L. pneumophila* isolates is providing such a high resolution genotyping method. However, *L. pneumophila* is difficult to isolate and isolation of outbreak strains often fails due to a viable but nonculturable (VBNC) state of the respective environmental population. Therefore, we developed a cultivation independent approach to detect single clones in drinking water. This approach is based on the extraction of DNA from drinking water followed by PCR using a set of 8 Variable Number of Tandem Repeats (VNTR) primer pairs necessary for MLVA genotyping of *L. pneumophila*. The PCR amplicons were analyzed by Single Strand Conformation Polymorphism (SSCP) and capillary electrophoresis to obtain the respective MLVA profiles. Parallel to the high resolution analysis, we used the same environmental DNA to quantify the number of *L. pneumophila* cells in the drinking water using real-time PCR with 16S rRNA targeted primers. We used a set of drinking water samples from a small scale drinking water network to test our approach. With these samples we demonstrated that the developed approach was directly applicable to DNA obtained from drinking water. We were able to detect more *L. pneumophila* MLVA genotypes in drinking water than we could detect by isolation. The developed approach could be a valuable tool to identify outbreak strains even after the outbreak has occurred and has the potential to be directly applied to clinical material.

5.2 Introduction

Legionella pneumophila is a gram-negative, facultative intracellular pathogen that accounts for the majority of cases of Legionnaire's disease in Europe (16). It is also the causative agent for a milder form of infection, the Pontiac fever (14). Legionellae are ubiquitous inhabitants of natural and man-made aquatic environments. They occur in bulk water and biofilms, where they replicate within protozoa which can serve as transmission vesicles and as protective shell against disinfection or heat treatment (2, 6, 7). In drinking water supply systems (DWSS), legionellae can survive in dead-end tubings, stagnated water in plumbings or seldom used facilities (2). The pathogen is transmitted via small droplets of water, e.g. aerosols from cooling towers, shower heads or air-conditions. In the human lung, it is able to enter and replicate within alveolar macrophages causing a severe pneumonia. Among the 48 species of the genus *Legionella*, (1, 4, 21, 22) *L. pneumophila* is responsible

for approximately 91% of all reported community-aquired cases of legionellosis. Among the 15 serogroups of *L. pneumophila*, serogroup 1 accounts for 84% of confirmed cases, as assessed by an international collaborative survey (40). Even among serogroup 1 isolates, a high genetic diversity has been observed by several studies (12, 30, 35).

Epidemiological analyses of infections caused by *L. pneumophila* depend on the accurate identification of strains, preferably at the clonal level. Therefore, several typing methods have been implemented in the last years, e.g. MLST (Multiple-locus sequence typing) which is based on DNA sequencing of multiple polymorphic DNA segments (13, 33). Recently, a multiple-locus variable-number of tandem repeat (VNTR) analysis (MLVA) was implemented by Pourcel et al. and approved by Eurosurveillance (30, 31). MLVA typing is used to determine the allele-related repeat size variation on different VNTR loci of *L. pneumophila* isolates. The method was further improved by adapting the eight-locus comprising MLVA analysis to capillary electrophoresis with the use of fluorescently labelled primers, thus providing a fast, reproducible and low-cost genotyping method for *L. pneumophila* isolates (26). Upstream isolation procedures face several problems: Especially in hot water with temperatures above 37°C, legionellae can loose culturability and enter a viable but nonculturable (VBNC) state (28). This VBNC state is mostly the reason why *L. pneumophila* cannot be isolated from aquatic environments which are suspected to be the source of infection (37). Additionally, cultivation of this fastidious bacterium is difficult due to slow growth and an overgrowing of plates by competing bacteria.

The aim of this study was to evaluate the utility of MLVA analyses directly on environmental DNA obtained from finished drinking water. Using DNA-based Single Strand Conformation Polymorphism (SSCP) analysis, this method should allow: (1) identification of strains that caused outbreaks, (2) monitoring of present *L. pneumophila* strains in a given sample at the clonal level without cultivation, and (3) sequence information on VNTR markers obtained directly from sequencing of SSCP gel bands, i.e. environmental DNA. To this end, we tested our approach with DNA from a set of drinking water samples from a small scale drinking water network. We demonstrated that the method provides a reliable tool for the analysis of samples where the number of present *L. pneumophila* cells is relatively low and isolation procedures did not succeed. Additionally, the complete sequence information of the VNTR-locus could be obtained from PCR amplicons separated on SSCP gels.

5.3 Materials and Methods

5.3.1 Strains and growth conditions

The reference strain *Legionella pneumophila subsp. pneumophila* strain Philadelphia DSMZ7513^T was provided by Molecular Diagnostics Center, Orihuela, Spain. All other reference strains (*L. pneumophila* strain Corby, *L. pneumophila* strain Lens, *L. jamestowniensis, L. jordanis, L. cincinnatiensis and L. feeleii*) were kindly provided by Michael Steinert from the institute of microbiology at the technical university of Braunschweig. All reference strains were grown on solid buffered charcoal yeast extract medium (BCYE) supplemented with L-cysteine and ferric pyrophosphate, with or without antibiotics (Oxoid, Basingstoke, UK).

5.3.2 Sampling of drinking water and isolation of *Legionella* spp. strains

Hot and cold drinking water samples were collected from several sources (for details see Tab. 1) on the campus of the Helmholtz Centre for Infection Research (HZI) in Braunschweig, Germany on June 23, 2009. In addition, cooling tower water samples (bulk water) were obtained on July 10, 2009. *Legionella* spp. strains were isolated from the samples according to ISO 11731-2 (monitoring method for the isolation and enumeration of *Legionella* organisms in water intended for human use). In brief, 10 - 1000 ml (mostly 250ml) of the water sample (or an appropriate dilution) were filtered onto a 0.45 µm HABG filter (mixed cellulose esters, black, with counting grid, Millipore, Schwalbach, Germany) and treated with 20 ml of acidic buffer (0.2 mol HCl/KCl – solution, pH 2.2) for 5min. After a washing step with 10 ml of sterile distilled water, the filter was transferred to GVPC (BCYE with glycine vancomycim polymixin cyclohexamide) or MWY (BCYE medium with antimicrobial agents, glycine, and differential dyes) solid medium (mibius, Düsseldorf, Germany) and incubated at 36 ± 1℃ for 3 to 10 days . *Legionella* colonies appeared as grey or white round shaped colonies with ground-glass opacity when observed after 3 days of culture. All *Legionella*-like colonies were picked from this medium and subcultured on BCYE (mibius, Düsseldorf, Germany) with and without antibiotics and sheep blood agar plates (mibius, Düsseldorf, Germany). Organisms growing on charcoal agar but not on blood agar were tested by PCR with *Legionella* genus specific primer pairs “Lgsp17F” and “Lgsp28R” (see below). Positive colonies were subcultured on BCYE medium and further characterized by complete sequencing of the 16S rRNA gene.

Tab. 1. Characteristics *of Legionella* sp. isolates obtained on the HZI.

Isolate designation	source of isolation	isolation date	isolation medium	L.pn.-PCR	dilution and isolation volume	16S rRNA gene sequence analysis (~1450bp amplicon)
SK 1	scullery, D-building	June 23, 2009	MWY	+	250ml	L.pn. strain Corby
SK 2	scullery, D-building	June 23, 2009	MWY	+	250ml	L.pn. strain Corby
Y 7	Y-building, technical room	June 23, 2009	MWY	+	250ml	L.pn. strain Corby
Y 8	Y-building, technical room	June 23, 2009	MWY	+	250ml	L.pn. strain Corby
GZ 1	GZ, toilet 3.015	June 23, 2009	MWY	-	250ml	L. anisa
GZ 2	GZ, toilet 3.015	June 23, 2009	MWY	-	250ml	L. anisa
KT 1	heat exchange water tower	July 10, 2009	GVPC	+	1:100 (250ml)	L. pn. Phil/L. pn. Paris
KT 2	heat exchange water tower	July 10, 2009	GVPC	+	1:100 (250ml)	L. pn. Phil/Paris/Corby
KT 3	heat exchange water tower	July 10, 2009	GVPC	+	1:100 (250ml)	L. pn. Phil/L. pn. Paris
KT 7	heat exchange water tower	July 10, 2009	MWY	+	1:100 (250ml)	L. pn. Phil/L. pn. Paris
KT 8	heat exchange water tower	July 10, 2009	MWY	+	1:100 (250ml)	L. pn. Phil/L. pn. Paris
KT 9	heat exchange water tower	July 10, 2009	MWY	+	1:100 (250ml)	L. pn. Phil/L. pn. Paris
KT 10	heat exchange water tower	July 10, 2009	MWY	+	1:100 (250ml)	L. pn. Phil/L. pn. Paris
KT 11	heat exchange water tower	July 10, 2009	MWY	+	1:100 (250ml)	L. pn. Phil/L. pn. Paris
KT 12	heat exchange water tower	July 10, 2009	MWY	+	1:100 (250ml)	L. pn. Phil/L. pn. Paris

For long-term storage of the drinking water samples and DNA extraction, bacteria were harvested from 3 - 5 l of drinking water by filtration through a filter sandwich consisting of a 0.2 µm pore size polycarbonate filter (90 mm diameter; Nucleopore; Whatman, Maidstone, United Kingdom) with a precombusted glass fiber filter on top (90 mm diameter; GF/F; Whatman) according to Eichler et al. (8). These filter sandwiches were stored at -70℃ until further analysis.

For enumeration of total bacteria, formaldehyde-fixed drinking water samples (2% final concentration) were stained with Sybr Green I dye (1:10000 final dilution; Molecular Probes, Invitrogen) for 15 min at room temperature in the dark. Five ml portions were filtered onto 0.2 µm pore size Anodisc filters (Whatman) and mounted with Citifluor on microscopic glass slides according the protocol of Weinbauer et al. (39). Slides were either analyzed directly with epifluorescence microscopy or stored frozen (-20℃) until examination. For epifluorescence microscopy, a microscope (Axioplan, Zeiss) with suitable fluorescence filters was used and the slides were examined using 100fold magnification. For each filter, either 10 photographs were taken and image sections of defined size (0.642 mm x 0.483 mm) were analyzed using the Image J software from MacBiophotonics (http://www.macbiophotonics.ca/) or 30 fields (0.125 mm x 0.125 mm) were counted by eye.

Heterotrophic plate counts (HPCs) were done in triplicate using an aliquot of the drinking water sample and the spread plate technique on R2A agar (Oxoid) plates. Incubation was carried out at two different temperatures according to the German drinking water regulation (36℃ for 48 h and 22℃ for 72 h) (10).

5.3.3 DNA extraction, PCR and real-time-PCR

For extraction of DNA from the filter sandwiches a modified DNeasy protocol (Qiagen, Hilden, Germany) was used. In brief, sandwich filters were cut into pieces, incubated with enzymatic lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2 % Triton X-100; ph 8,0) containing 10 mg/ml lysozym (Sigma) for 60 min in a 37°C water bath. After addition of AL-buffer from the kit, the samples were incubated at 78°C in a shaking water bath for 20 min. After filtration through a polyamide mesh with 250 µm pore size, absolute ethanol was added to the filtrate (ratio filtrate/ethanol 2:1) and the mixture was applied to the spin-column of the kit. After this step, the protocol followed the manufacturer's instructions. DNA was eluted from the column with DNase/RNase free water and stored at -20°C. Quantification of DNA was carried out using Picogreen (dsDNA quantification, Molecular Probes; Invitrogen) according to Weinbauer and Höfle (39). For *Legionella* spp. isolates genomic DNA was isolated from agar plates using the DNeasy standard procedure for gram-negative bacteria (Qiagen, Hilden, Germany).

Different PCR reactions were carried out as in the following: For the classification of *Legionella*-like colonies obtained from the HZI sampling, a *Legionella* genus-specific PCR using the primer pair Lgsp17F (5´-GGCCTACCAAGGCGACGATCG -3´) and Lgsp28R (5 - CACCGGAAATTCCACTACCCTCTC-3´) and a *Legionella pneumophila* specific PCR using the Primer pair Lp-16S_246-248F (CCTGGGCTTAACCTGGGAC) and Lp-16S_246-248R (CTTAGAGTCCCCACCATCACAT) were applied. For sequencing of the complete 16S rRNA gene of *Legionella* isolates, the primer pair "27F" (AGAGTTTGATCMTGGCTCAG) and "1492R" (TACGGYTACCTTGTTACGACTT) was used. Each amplification was carried out using 0.2 to 2 ng of DNA template in a final volume of 50 µl under the conditions given in Tab. 2. Amplification was achieved using HotStarTaq DNA polymerase (QIAGEN, Hilden, Germany).

Tab. 2. PCR programs used in this study.

	initial denaturation	denaturation	annealing	elongation	final elongation
Legionella genus-specific	95°C 15min	95°C 30s	66.5°C 30s	72°C 30s	72°C 10min
Legionella pneumophila	95°C 15min	95°C 45s	60.0°C 45s	72°C 45s	72°C 20min
Legionella 16S rRNA gene	95°C 15min	95°C 90s	55.0°C 40s	72°C 90s	72°C 10min

For quantification of the number of *Legionella pneumophila* genes in drinking water samples, real-time PCR using SybrGreen I chemistry (Roche Diagnostics, Germany) was implemented. For real-time PCR the *Legionella pneumophila* specific primer pair Lp-16S_246-248 (see above) was used. On the Light Cycler 480 real-time PCR machine (Roche Diagnostics, Germany) the 16S rRNA gene was used to determine the number of

L. pneumophila genomes in the approach utilizing a standard of genomic DNA from *L. pneumophila* strain Philadelphia (DSMZ7513^T) and a detection limit of approximately 0.4 *Legionella pneumophila* cell per PCR assay.

5.3.4 MLVA-8 and capillary electrophoresis

MLVA-8 single PCR reactions were carried out using the previously described primer sets of Pourcel et al. (31) and the diagram given in Fig. 1. PCR reactions of 50 µl contained in end concentrations: 1 x reaction buffer (Qiagen, Hilden, Germany), 2.5 U of HotStarTaq DNA polymerase, 0.2 - 2 ng of template DNA, 125 nmol $MgCl_2$, 7.5 nmol of each deoxynucleoside triphosphate (dNTP) and 0.02 nmol of each primer. Forward primers for PCR were synthesized with a biotin-tag on the 5´- end (MWG Operon, Ebersberg, Germany). Each PCR was carried out using an initial denaturation step for 15 min at 95℃, a total of 35 cycles (30 s at 95℃, 30 s at 65℃, and 30 s at 72° C) followed by a final elongation for 10 min at 72℃.

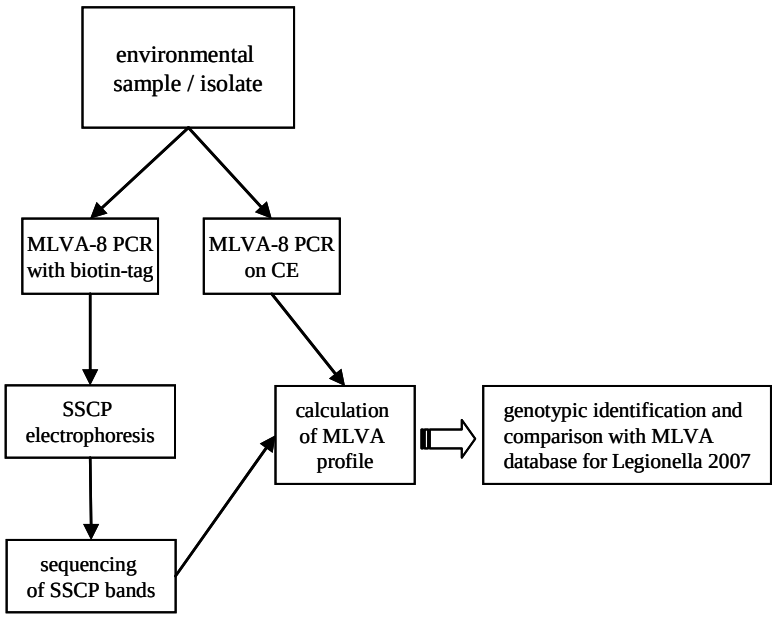


Fig. 1. Flow chart of the analytical setup. Either an isolate or an environmental sample is analyzed simultaneously by MLVA-8 for capillary electrophoresis (CE) and Single Strand Conformation Polymorphism (SSCP) electrophoresis. For CE, PCR was done using fluorescently labelled primers to enable determination of the repeat numbers in the alleles through analysis of the peaks in the electropherograms. Subsequently, a comparison with the MLVA database for *Legionella* 2007 was conducted (http://bacterial-genotyping.igmors.u-psud.fr/Legionella2006/help.htm) to identify single clones. For the SSCP analysis of the samples, PCR amplification with 5´-biotin tagged forward primers was applied. Bands originating from the SSCP fingerprints were cut out and analyzed by sequencing. The sequence information could then be converted into the repeat profile.

Tab. 3. *Legionella pneumophila* MLVA-8 setup for capillary electrophoresis.

Primer set	Dye	Color	Panel	Repeat length in bp	Total flanking region in bp
Lpms1b	VIC	Green	I	45	205
Lpms3	FAM	Blue	I	96	173
Lpms33	NED	Yellow	I	125	102
Lpms35	PET	Red	I	18	148
Lpms13	NED	Yellow	II	24	164
Lpms17	VIC	Green	II	39	200
Lpms19b	FAM	Blue	II	21	89
Lpms34	PET	Red	II	125	84

In the PCR for MLVA-8 analysis for capillary electrophoresis, forward primers were labeled with VIC, PET, FAM and NET (Applied Biosystems, Foster City, CA), see Tab. 3. All reverse primers were synthesized unlabelled (MWG Operon, Ebersberg, Germany). After amplification, 1 µl (PCR for isolates) or up to 10 µl (*in situ* samples) of the PCR products of Lpms 1b (here:1), 3, 33 and 35 (Panel I) or Lpms 13,17, 19b (here 19) and 34 (Panel II) were pooled, purified using the MinElute PCR Clean-up Kit (Qiagen, Hilden, Germany) and diluted 1:50 or 1:100 with distilled water. In the wells of a 96 well microtiter plate, 1 µl of the pooled and diluted PCR product mix was added to 8.85 µl of HiDi (Highly deionized) Formamide (Applied Biosystems) and 0.15 µl GeneScan 1200LIZ size standard (Applied Biosystems) containing 68 single-stranded labeled fragments in the range of 20 to 1200 bp. The samples were denatured for 3 min at 95°C in a thermoblock, cooled on ice for at least 1min and spun briefly for 500 rpm in a Multifuge 1 centrifuge (Heraeus, Germany). Fragment analysis was performed on a 3130xl sequencer (Applied Biosystems) equipped with 50 cm capillaries, using POP-7 polymer, with the recommended running parameters for the GeneScan LIZ1200 size standard (running period: 2.5 h, running voltage: 8.5 kV, injection voltage: 15 kV). Each Lpms locus was identified by color and assigned a size by the GeneMapper software version 3.7 (Applied Biosystems) using settings for microsatellite analysis. The number of repeats in the alleles was estimated by subtracting the invariable flanking region from the amplicon size divided by the repeat unit length, as determined for reference strain Philadelphia according to Pourcel et al. (31).

5.3.5 Single-strand-conformation polymorphism (SSCP) electrophoresis

For the preparation of ssDNA from the PCR amplicons a variant of the protocol described by Eichler et al. (9) was applied. Briefly, magnetic streptavidin coated beads (Promega, Madison, Wis.) were used to prepare ssDNA from the PCR amplicons. For SSCP analysis, 25 ng of the obtained ssDNA was mixed with gel loading buffer (95% formamide, 10 mM NaOH, 0.25% bromphenol blue, 0.25% xylene cyanol) in a final volume of 7 µl. After incubation for 3 min at 95°C, the ssDNA samples were stored on ice, loaded onto a

nondenaturing polyacrylamide-like gel (0.6 x MDE gel solution; Cambrex BioScience, Rockland, Maine) and electrophoretically separated at 20℃ at 400 V for 20 h on a Macrophor sequencing apparatus with 20 cm or 55 cm glass plates (Pharmacia Biotech, Germany). The gel was silver stained according to Bassam et al.(3). Dried SSCP gels were digitized using an Epson Expression 1600 Pro scanner.

5.3.6 Reamplification and sequencing of ssDNA bands from SSCP gels

Sequence information was obtained following the protocol of Eichler et al. (9). Briefly, ssDNA bands were excised from the SSCP acrylamide gels, and boiled in Tris buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM KCl, 0.1% Triton X-100, pH 9). 5 µl of the solution were used in a reamplification PCR with the unbiotinylated Lpms primers described above. After checking on a 2% agarose gel, the amplicons were purified and subsequently sequenced by cycle sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems, Foster City, CA). Before analysis on an ABI Prism 3100 Genetic Analyzer, the products were purified using the BigDye Terminator purification kit (Qiagen, Hilden, Germany). The obtained sequence information was used to calculate Lpms locus repeats comparable to the data obtained from capillary electrophoresis (see above). The partial 16S rRNA gene sequences retrieved from the genus-specific screening are accessible at the GenBank/EMBL/DDBJ accession numbers GU598175 - GU598211. Sequences retrieved from SSCP gels of strain-specific MLVA profiles are deposited under accession numbers GU598121 (band number 1) - GU598174 (bend number 54).

5.4 Results

5.4.1 Seasonal variation of *Legionella* spp. in drinking water as assessed by SSCP fingerprinting

During one and a half year the drinking water from the tap of room D0.04 was analyzed using *Legionella* genus-specific SSCP-fingerprints based on a 424 bp PCR amplicon (Fig. 2). We observed a high variation of different *Legionella* species throughout the seasons with a slightly higher variability in spring and autumn. Compared to reference strains which showed only one distinct band (a second band, like in the Philadelphia strain, is another conformation of the same ssDNA, as confirmed by sequencing), the fingerprints showed rather diverse patterns indicating several *Legionella* species on one sampling date. Sequencing of various bands resulted in information up to the species level (see Supplementary Material Tab. S1). Most of the species were identified with 97% - 100% sequence similarity as uncultured members of the family *Legionellaceae* (*Legionella, Fluoribacter and Tatlockia*) but some sequences could be clearly identified as *Legionella pneumophila* genotypes.

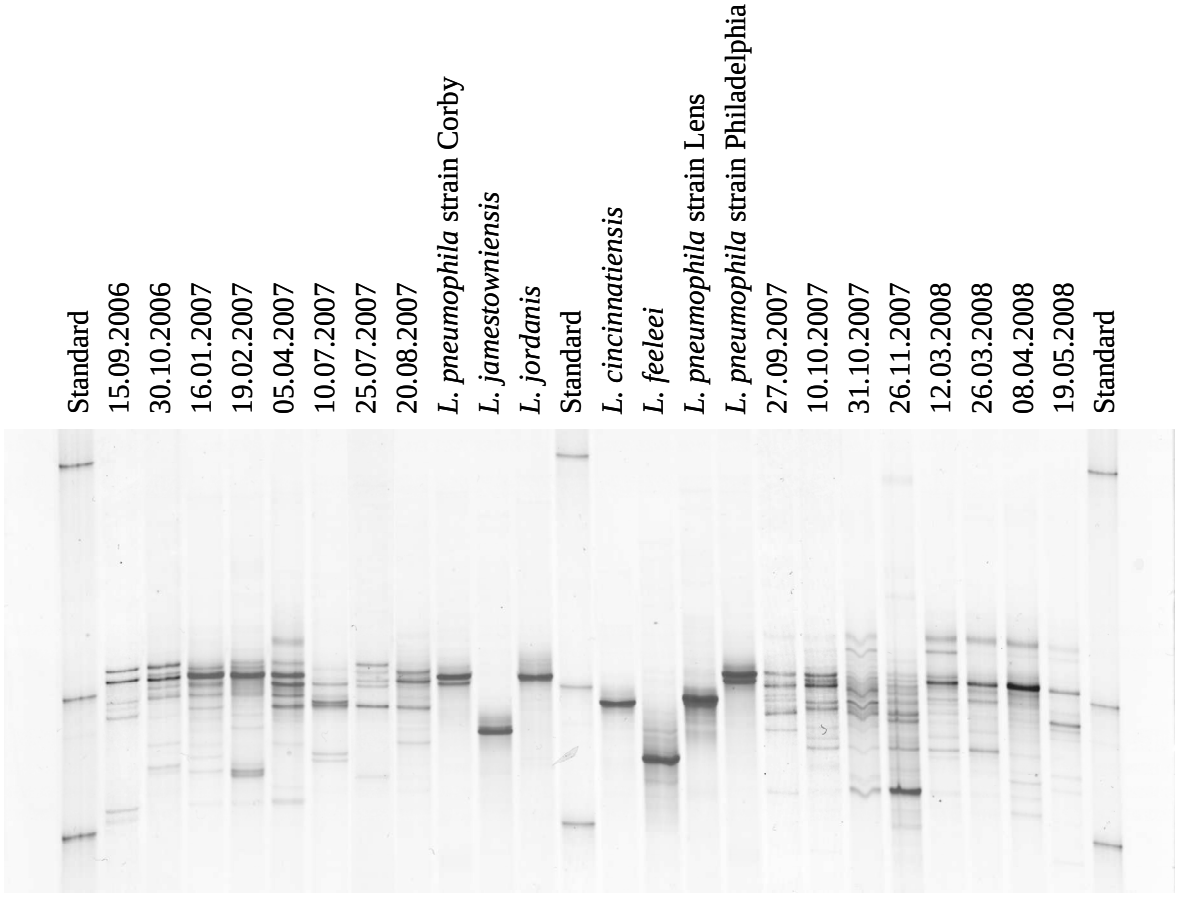


Fig. 2. Single-strand conformation polymorphism (SSCP) fingerprints of *Legionella* genus-specific screening. DNA of drinking water, sampled on the dates indicated, was extracted from frozen filters and genus-specific PCR was performed with primer pair "Lgsp17F" and "Lgsp28R" (see material and methods). In the centre of the gel, single stranded PCR products from 7 reference strains were analysed. Sequences retrieved from this SSCP gel are further characterized in supplementary figure 1 and supplementary table 1. Standard: species standard of three different species for calculation of running distances.

5.4.2 Real-time PCR for quantification of *L. pneumophila* cells

To estimate the abundances of single genotypes in our drinking water samples and to determine the efficiency of the developed assay, we used a *Legionella pneumophila* specific real-time PCR approach for quantification. We compared the numbers of genomes with heterotrophic plate counts on R2A medium, total cell counts counted by epifluoerescence microscopy and the numbers of colonies visible on the filters used for isolation of *Legionella* cells (Tab. 4). Heterotrophic plate counts at 36℃ resulted in moderate CFU counts 4-6 logs lower then the cell numbers counted by epifluorescence microscopy. At 20℃ we even observed less CFUs per litre sample volume. On the filters, used for the isolation of *Legionella* spp. strains, we could only observe few numbers of grey-white colonies with ground-glass opacity indicating *Legionella*-like bacteria. By real-time PCR, we obtained

numbers ranging from 0.7 to 89.9 *Legionella pneumophila* cells per litre of sample volume. The highest number was detected in the infrequently used men's shower in the D-building and the lowest numbers in the two cold water samples (< 5). Due to a rather low number of *Legionella* cells in the samples, as detected by real-time PCR, single colonies observed on the agar plates can lead to an overestimation of *Legionella* CFUs.

Tab. 4. Characteristics of sampling sites used in this study. Total bacterial cell numbers of the drinking water as determined by epifluorescence microscopy using Sybr Green I staining of formaldehyde fixed samples. Heterotrophic plate counts (HPC) on R2A agar and *Legionella* counts on BCYE agar were determined by the spread plate technique and an appropriate aliquot of sample volume. *L. pneumophila* cells were detected by real-time PCR using the *L. pneumophila* specific primer pair Lp-16S_246-248.

type of drinking water	site	total cell counts/litre	HPC on R2A 36°C / 48h CFU/litre	HPC on R2A 22°C / 72h CFU/litre	*Legionella*-like colonies on BCYE/litre	*Legionella pneumophila* cells/litre detected by real-time PCR
hot water	boiler house	$1,89x10^8$	$3,00x10^2$	0	0	5,1
	men´s shower, D-building	$1,47x10^8$	$6,67x10^3$	$7,00x10^2$	0	89,9
	scullery, D-building	$1,58x10^8$	$2,30x10^3$	0	6,7	3,8
cold water	room D0.04, D-building	$2,57x10^8$	0	$7,00x10^2$	0	1,8
	Y-building, technical room	$1,95x10^8$	$1,70x10^4$	n.d.	6,7	0,7

5.4.3 Isolation record of *L*. spp. strains from a small scale drinking water network

Based on our observations from the genus-specific screening of our drinking water from the tap, we investigated our small-scale drinking water network on the HZI campus regarding the occurrence of *Legionella* species. We were able to isolate different *Legionella*-like colonies on BCYE agar and tested them with *Legionella* genus-specific and *Legionella pneumophila* specific primers. These isolations resulted in 15 *Legionella* spp. strains which were further characterized by sequencing of the complete 16S rRNA gene (see Tab. 1). Four isolates could be clearly assigned to *Legionella pneumophila* strain Corby (SK 1 and 2 and Y 7 and 8), two as *Legionella anisa* (GZ 1 and 2) and all nine cooling tower isolates to *Legionella pneumophila*. Due to the high sequence similarity of the 16S rRNA gene in the genus *Legionella* (32), no taxonomic information at the clonal level could be obtained here.

5.4.4 MLVA-8 CE of reference strain and environmental isolates

We determined the clonal genotypes of our isolates and the reference strain Philadelphia according to the well-established MLVA-8 analysis developed by Pourcel et al. (30, 31). These genotypes were determined using capillary electrophoresis (CE) to assess the PCR fragment size estimation. On Fig. 3 a, b (left side) the electropherograms of the two Panels are shown for isolate SK 1 and peaks can be clearly identified for all eight Lpms markers. Based on the PCR product sizes the number of repeats in the alleles were

calculated by subtracting the number of flanking bases and division by the repeat unit length. The observed allele sizes (by CE) were also compared with the sizes reported in the “Help file for the Legionella pneumophila MLVA typing page” (http://bacterial-genotyping.igmors.u-psud.fr/Legionella2006/help.htm). This file should assist the assignment when calculating allele sizes by gel-based MLVA. However, we observed only minor differences in size to our CE-based results for the reference strain and our isolates. This did not lead to a false prediction of alleles for all VNTR markers. All four isolates from the rinsing room for laboratory material (scullery) of the D-building and the technical room of the Y-building showed exactly the same MLVA genotype (Tab. 5, upper part). In addition, all cooling tower isolates showed nearly the same allelic profile with only a small difference for Locus 17, where no PCR product was obtained for the isolates KT 8 and KT 10.

5.4.5 *In situ* MLVA-8 CE of environmental samples

We also performed the MLVA-8 capillary electrophoresis analysis directly on the DNA samples from our sampling and were able to detect the most markers in every sample. We did not get any PCR product in the D0.04-sample for Lpms 3 and 35 (see Tab. 6). The electropherograms of these “*in situ*” MLVA also had very high background fluorescence and were not easy to analyze (see scullery *in situ*, right side Fig. 3 c, d). Nevertheless we obtained more than one single peak for some samples. For the scullery *in situ* sample for example, we detected one (Lpms 1) or two (Lpms 13) additional peaks corresponding to other repeat sizes (Fig. 3) in the sample. Due to the difficulties with direct MLVA-8 CE on environmental DNA (e.g. high background, no possibility for direct sequencing) we applied single-strand conformation polymorphism (SSCP) gel electrophoresis for the analysis of these complex samples. This method showed a high resolution separation (9) and allowed sequencing of the separated amplicons to determine precisely the MLVA profile.

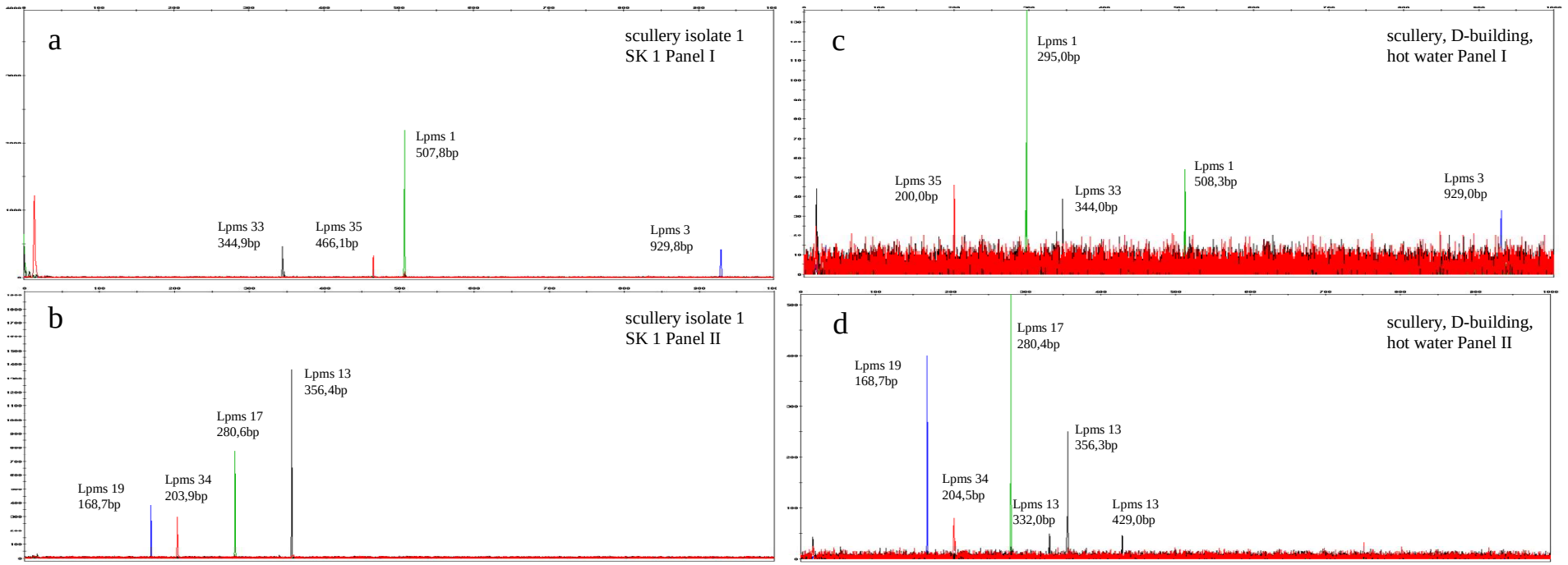


Fig. 3. Representative electropherograms of the MLVA-8 PCR products separated by capillary electrophoresis (CE) and identified according to their sizes and colours. The electropherograms correspond to the PCR products from the *Legionella pneumophila* scullery isolate SK 1 (a, b, left electropherograms) and the *in situ* MLVA of DNA from the scullery sample (c, d, right electropherograms). Panel I: Lpms 1 (VIC [green]), Lpms 3 (FAM [blue]), Lpms 33 (NED [black]), Lpms 35 (PET [red]); Panel II Lpms 13 (NED), Lpms 17 (VIC), Lpms 19 (FAM), Lpms 34 (PET).

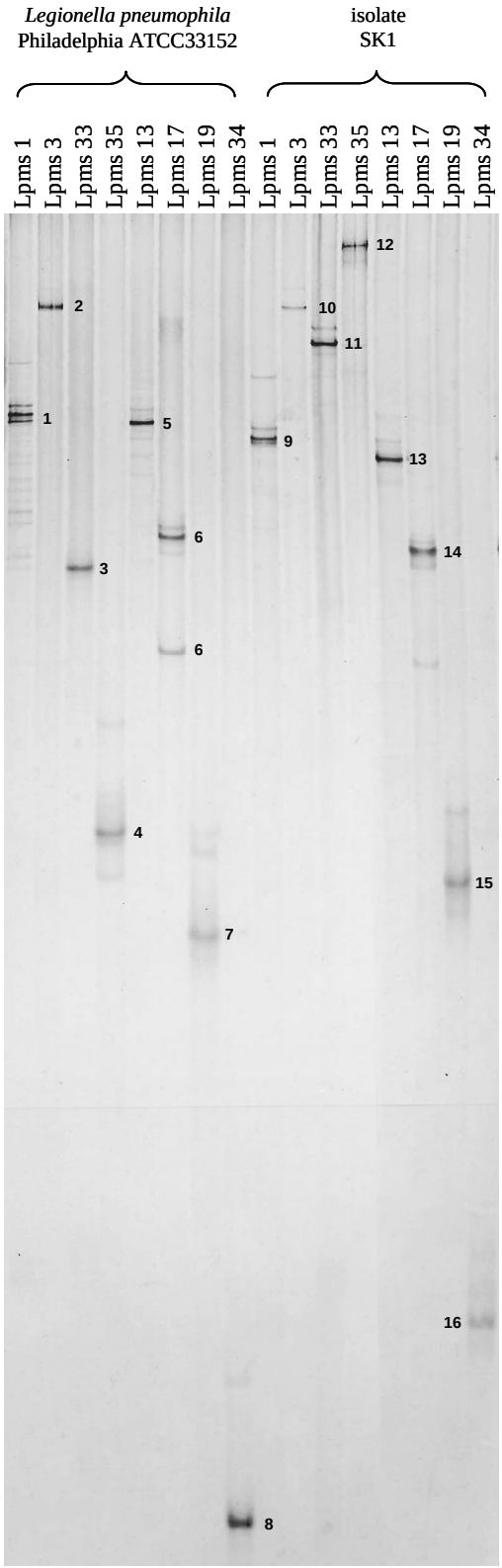
5.4.6 SSCP gel electrophoresis of strain-specific MLVA profiles

We wanted to apply the MLVA-8 analysis directly to environmental DNA for the specific identification of different *L. pneumophila* genotypes in a single sample without prior isolation. To obtain better resolution than CE, we tested to run the single amplicons from the MLVA-8 on a single-strand conformation polymorphism (SSCP) gel. This sensitive high-resolution method would be able to show different amplicon sizes and different sequences (i.e. the different allele sizes) on one gel with the option to sequence the amplicons. For testing the approach, we amplified all eight loci and prepared the single-stranded amplicons for SSCP analysis for a reference strain and one isolate (SK 1, Fig. 4a). In every lane one clear major band is dominating with only a few exceptions (e.g. Lpms 1). These double bands occur probably due to binding of a primer to the inner repeat region of the locus as presumed by Nederbragt et al. (26). To confirm the developed approach, we sliced out all major bands from the gel, reamplified them with the respective primer pair and sequenced the bands. For some additional bands (e.g. Lpms 1) we were not able to obtain a PCR product supporting the assumption mentioned above (data not shown). All other products resulted in the same repeat sizes than those obtained from capillary electrophoresis. A minor limitation of the direct sequencing was the product length. We were therefore not able to sequence the complete product of the Lpms 3 locus because it was too long (~930 bp) to be read completely in our sequencing approach.

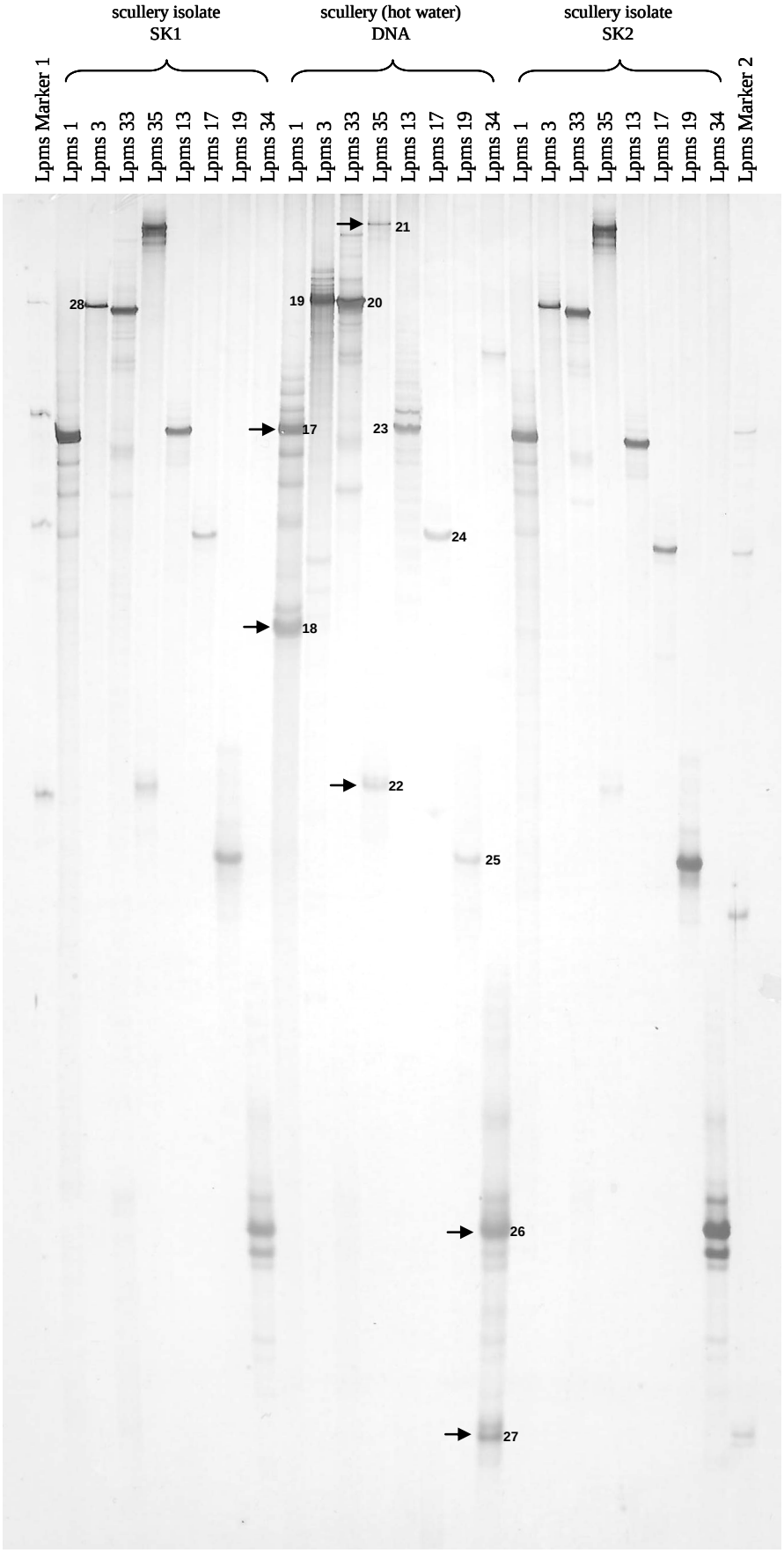
5.4.7 SSCP gel electrophoresis of MLVA-8 PCR products from environmental DNA

We performed the same analysis of the single loci directly on two isolates (here SK 1 and SK 2) and the corresponding DNA sample extracted from the source of the isolates, i.e. the scullery bulk water, to test if the SSCP fingerprinting was working directly on our environmental DNA (Fig. 4b). On this gel, we used the amplicons of Panel I and Panel II MLVA-PCR from the Philadelphia strain as single-strand marker on the outside (Marker I = Panel I, Marker 2= Panel II). The two isolates are showing distinct major bands for all loci corresponding to the gel shown in Fig 4a (SK 1). For the environmental DNA from the scullery we were able to detect more than one major band per loci for loci Lpms 1, Lpms 35 and Lpms 34. When calculating the repeats from the sequences (see Tab. 5) we observed that only the two additional bands from the Markers Lpms 1 and Lpms 35 were also corresponding to other repeat sizes. For Lpms 1, the detected repeat size was exactly the same also detected with *in situ* capillary electrophoresis. The two different major bands of Lpms 34 appeared to have nearly the same sequence.

4a.


Legionella pneumophila
Philadelphia ATCC33152
isolate
SK1
Lpms 1
Lpms 3
Lpms 33
Lpms 35
Lpms 13
Lpms 17
Lpms 19
Lpms 34
Lpms 1
Lpms 3
Lpms 33
Lpms 35
Lpms 13
Lpms 17
Lpms 19
Lpms 34
1
2
3
4
5
6
6
7
8
9
10
11
12
13
14
15
16

4b.


scullery isolate SK1
scullery (hot water) DNA
scullery isolate SK2
Lpms Marker 1
Lpms 1
Lpms 3
Lpms 33
Lpms 35
Lpms 13
Lpms 17
Lpms 19
Lpms 34
Lpms 1
Lpms 3
Lpms 33
Lpms 35
Lpms 13
Lpms 17
Lpms 19
Lpms 34
Lpms 1
Lpms 3
Lpms 33
Lpms 35
Lpms 13
Lpms 17
Lpms 19
Lpms 34
Lpms Marker 2
28
19
20
21
17
23
24
18
22
25
26
27

4c.

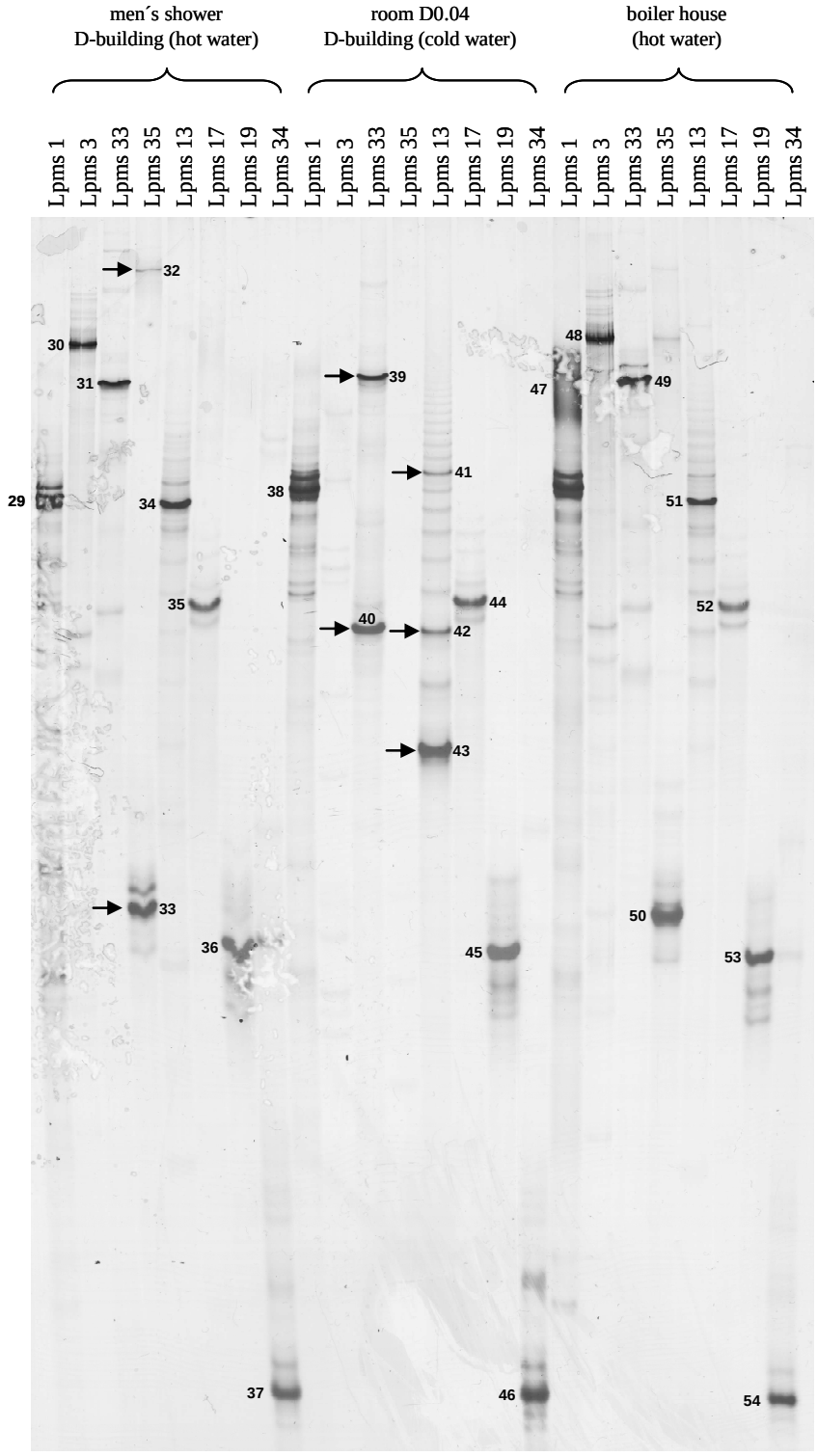


Fig. 4. **(a)** Single-strand conformation polymorphism (SSCP) gel electrophoresis of strain-specific MLVA profiles: *Legionella pneunmophila* strain Philadelphia and isolate SK 1. Applied are single-stranded amplicons of all eight MLVA loci for the reference strain and SK 1. **(b)** SSCP gel electrophoresis of MLVA profiles: isolates SK 1 and SK 2 and the corresponding environmental DNA. **(c)** SSCP gel electrophoresis of MLVA-8 profiles of different environmental DNA samples. Band numbers (BN) represent sequences deposited in the GenBank database with the accession numbers given in material and methods.

Tab. 5. MLVA-8 profiles for *L. pneumophila* isolates and in situ samples using CE. -1 means: no PCR product.

isolate designation or in-situ analysis	sampling site or isolate source	Lpms locus - typing profile							
		1	3	13	17	19	33	34	35
L.pn. Philadelphia		8	8	11	2	4	1	1	3
SK 1	scullery, D-building	7	8	8	2	4	2	1	18
SK 2	scullery, D-building	7	8	8	2	4	2	1	18
Y 7	Y-technical room	7	8	8	2	4	2	1	18
Y 8	Y-technical room	7	8	8	2	4	2	1	18
KT 2	cooling tower	7	7	10	2	4	3	1	17
KT 3	cooling tower	7	7	10	2	4	3	1	17
KT 1	cooling tower	7	7	12	2	4	3	1	17
KT 7	cooling tower	7	7	12	2	4	3	1	17
KT 8	cooling tower	7	7	12	-1	4	3	1	17
KT 9	cooling tower	7	7	12	2	4	3	1	17
KT 10	cooling tower	7	7	12	-1	4	3	1	17
KT 11	cooling tower	7	7	12	2	4	3	1	17
KT 12	cooling tower	7	7	12	2	4	3	1	17
MD in-situ	men´s shower	7	8	8	2	4	2	1	3
D004 in-situ	D004, D-building	7	-1	11	2	4	1	1	-1
KH in-situ	boiler house	7	8	8	2	4	2	1	3
KH in-situ	boiler house	7	8	11	2	4	2	1	3
KH in-situ	boiler house	7	8	8	2	4	2	2	3
KH in-situ	boiler house	7	8	11	2	4	2	2	3
KH in-situ	boiler house	4	8	8	2	4	2	1	3
KH in-situ	boiler house	4	8	11	2	4	2	1	3
KH in-situ	boiler house	4	8	8	2	4	2	2	3
KH in-situ	boiler house	4	8	11	2	4	2	2	3
SK in-situ	scullery, D-building	7	8	8	2	4	2	1	3
SK in-situ	scullery, D-building	7	8	11	2	4	2	1	3
SK in-situ	scullery, D-building	7	8	3	2	4	2	1	3
SK in-situ	scullery, D-building	2	8	8	2	4	2	1	3
SK in-situ	scullery, D-building	2	8	11	2	4	2	1	3
SK in-situ	scullery, D-building	2	8	3	2	4	2	1	3

We analysed additional environmental samples with our SSCP fingerprinting approach. In Fig 4 c, the Lpms products for two hot water samples (men's shower, boiler house) and a cold water sample (D0.04) are shown. Except of the markers Lpms 3 and 35, which we also could not detect by CE in this case, we were able to get amplicons and ssDNA for all other markers and samples. Again, we sequenced major bands from the gel resulting in similar repeat information as gathered from the capillary electrophoresis. Except of Lpms 1, where we expected several other bands due to additional binding of primers, we observed only in the D0.04 sample several additional bands. Through sequencing of the three major Lpms 13 amplicons, we found that these corresponded to three different alleles for this marker (Fig. 4 c and Tab 6), namely three, five and eleven repeats. For all other loci we could not observe additional alleles by sequencing of additional bands. For example for Lpms 19 in the boiler house sample, all three major bands exhibited the same sequence. Therefore we decided to sequence only the major bands of the SSCP MLVA-8 fingerprints. For the *in situ* samples, all possible genotypes were calculated based on the different results from CE and SSCP sequencing (Tab. 5, lower part).

Tab. 6. Comparison of MLVA-8 profiles determined by CE and SSCP analysis for the isolates and the *in situ* samples. n.d. no PCR product; #, sequence information too short for analysis.

		Lpms Locus							
strain/sample	Method	1	3	13	17	19	33	34	35
L. pn. Philadelphia	CE	8	8	11	2	4	1	1	3
	SSCP	8	#	11	2	4	1	1	3
L. p . SK 1	CE	7	8	8	2	4	2	1	18
	SSCP	7	#	8	2	4	2	1	18
1	CE	7	8	8	2	4	2	1	3
scullery in-situ	SSCP	7	#	8	2	4	2	1	3
additional VNTR	CE	2		7, 11					
scullery in-situ	SSCP	2							>16
2	CE	7	8	8	2	4	2	1	3
men´s shower in-situ	SSCP	7	#	8	2	4	2	1	3
additional VNTR	CE								
men´s shower in-situ	SSCP								18
3	CE	7	n.d.	11	2	4	1	1	n.d.
D004 in-situ	SSCP	7	n.d.	11	2	4	1	1	n.d.
additional VNTR	CE								
D004 in-situ	SSCP			3, 5			2		
4	CE	7	8	8	2	4	2	1	3
boiler house in-situ	SSCP	7	#	8	2	4	2	1	3
additional VNTR	CE	4		11					
boiler house in-situ	SSCP	4							

5.5 Discussion

Legionella pneumophila can pose a significant health threat for humans if present in man-made aquatic environments, most notably to immunocompromised persons (37). To understand infections by this pathogen, especially regarding its epidemiological aspects, an identification of strains at the subspecies level is necessary. Molecular tools based on the analysis of bacterial DNA, such as MLST or MLVA, have become widely accepted in molecular typing studies of pathogenic bacteria (15, 17, 18, 24). The MLVA analysis is based on polymorphic minisatellites (VNTRs) on different loci, where recombination and DNA polymerase slipping often happen. If occurring with certain frequencies, these events can result in changes of the repeat sizes between different strains at a given locus (38). Currently, MLVA data for *L. pneumophila* and several other pathogens such as *Mycobacterium tuberculosis* or *Pseudomonas aeruginosa* can be obtained from the central website http://minisatellites.u-psud.fr/ and the amount of data is increasing daily (19). Recently, Pourcel et al. (30, 31) developed a MLVA-8 gel-based typing profile for *Legionella pneumophila.* Nederbragt et al. (26) were able to transfer this method to capillary electrophoresis (CE) for an improved typing of *L. pneumophila* isolates from patients or the environment. Nevertheless, partly due to the VBNC state of *Legionella*, the isolation of *L. pneumophila* from environmental (bulk water, biofilms, etc) or clinical samples (sputum, lung biopsies, etc.) poses a great challenge (28, 36).

We monitored our drinking water for members of the genus *Legionella* using cultivation-independent molecular techniques during one and a half year. We observed a high variation of species throughout the seasons and sequence information was obtained from several bands of the SSCP gel. Most of the detected species were uncultured members

of the family *Legionellacea*. But we also detected 16S rRNA sequences of pathogenic species e.g. *Legionella pneumophila* strain Corby, with high likeliness (99% sequence similarity). As the minimum infectious dose for a severe infection with *L. pneumophila* is not known exactly (27) and highly depends on the susceptibility of the exposed person (34), we chose to quantify the detected *L. pneumophila* cells in a culture-independent way using specific real-time PCR. We detected only low numbers (0.7 - 1.8 *L. pneumophila*. cells/l bulk water) in the cold water samples, but relatively high numbers in the hot water samples (up to 90 cells/l) especially in the seldom used men's shower. This is in accordance with several studies, where *Legionella* is predominantly isolated from hot water sources such as dead-end tubes of the mains or infrequently used taps (23). In a German study of hot water samples from 452 households, water temperature was observed as probably the most important factor for multiplication of legionellae (25). Furthermore, it has been investigated that in many outbreaks of Legionnaire's disease, hot water was the most frequently involved source of infection (5, 11, 20, 29).

To bridge the gap to cultivation we characterized 15 *Legionella* strains which we obtained by sampling of different points on the HZI campus. By 16S rRNA gene sequencing, 13 strains could be identified as *L. pneumophila*. The rather low number of isolates could be explained by the VBNC state of the bacteria, their intracellular occurrence in their natural protozoan host and accompanying microflora complicating the acquisition of isolates. We genotyped the 13 *L. pneumophila* isolates with the MLVA method described by Nederbragt et al. (26) using capillary electrophoresis (CE). VNTR analysis of the eight loci resulted in three different MLVA-8 genotypes. When analysing our environmental DNA with CE, we had to face several problems like PCR inhibition resulting in low amplification rates as well as high background signals complicating the analysis of these complex samples. Therefore, a calculation of repeats for the VNTR loci from CE data was not always feasible. To overcome these analytical problems, we chose single strand conformation polymorphism (SSCP) gel electrophoresis which we previously applied for the genus-specific screening. Using a longer gel plate for the separation of single stranded PCR fragments we were able to detect fragments in a size range from 90 - 1000 bp. This range was suitable for the separation of all single-stranded tandem repeat locus amplicons from the MLVA-8 assay. Using a reference strain and some of our *L.pneumophila*. isolates, we demonstrated that the method gave clear and informative results for every of the eight MLVA loci described by Nederbragt et al. (26). Through sequencing of the single bands of the SSCP gel, we showed that not only the length of the PCR product but also the complete underlying sequence information could be determined by this method from environmental DNA. Optimisation of the PCR, like touchdown PCR, could minimize unspecific binding of the primers in the middle part of the repeats as applied by Nederbragt et al. (26).

After adapting our approach to environmental DNA, we could identify MLVA-genotypes in samples without isolation success (e.g. for the D0.04 sample we were not able to isolate legionellae) and additional MLVA profiles based on different alleles for one marker (e.g. Lpms 13, D0.04). By sequencing we confirmed that additional SSCP bands for one VNTR locus derived from different alleles. We tested our approach with additional DNA extracts from hot and cold water and could obtain clear bands for every MLVA locus. We assume that problems in the detection of one locus can arise if the concentration of *L. pneumophila* cells per litre would decrease under a detection limit of 2 cells/litre because we had problems to obtain PCR products for one or two of the eight loci in the D0.04 sample where we detected only 1.8 cells/litre of finished drinking water. We were not able to obtain *in situ* MLVA results when the concentration was even lower (Y-building, data not shown). Other studies dealing with the quantification of few pathogens in environmental samples are facing the same problem. Nevertheless, we think that the approach is very sensitive and can detect additional genotypes even if cells are in low concentrations as they are with certainty in finished drinking water samples where these kinds of pathogens are normally rare. Optionally, specific VNTR loci could be quantified by locus - specific real-time PCR. A subsequent melting curve analysis would then provide detailed information about the abundance of single genotypes in a given environmental sample.

To our knowledge, this is the first study applying the MLVA approach directly to environmental DNA samples such as drinking water. We think that the developed approach could help identifying outbreak strains long after the outbreak has occurred if the DNA or the water samples have been preserved for later analysis. In addition, this approach could also be applied to clinical samples without cultivation of the infective strains and contributes thereby to an improved surveillance of Legionnaires' disease.

5.6 Acknowledgments

This work was supported by funds from the European Commission for the HEALTHY WATER project (FOOD-CT-2006-036306). The authors are solely responsible for the content of this publication. It does not represent the opinion of the European Commission. The European Commission is not responsible for any use that might be made of data appearing therein. We thank Manuela Hölscher and Julia Strömpl for outstanding technical support.

5.7 References

1. **Adeleke A. A., B. S. Fields, R. F. Benson, M. I. Daneshvar, J. M. Pruckler, R. M. Ratcliff, T. G. Harrison, R. S. Weyant, R. J. Birtles, D. Raoult, and M. A. Halablab**. 2001. *Legionella drozanskii* sp. nov., *Legionella rowbothamii* sp. nov. and *Legionella fallonii* sp. nov.: three unusual new *Legionella* species. Int. J. Syst. Evol. Microbiol **51**:1151-1160.

2. **Atlas R. M.** 1999. *Legionella*: from environmental habitats to disease pathology, detection and control. Environ. Microbiol **1**:283-293.

3. **Bassam B. J., G. Caetano-Anoll, and P. M. Gresshoff**. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. **196**:80-83.

4. **Benson R. F., and B. S. Fields**. 1998. Classification of the genus *Legionella*. Semin. Respir. Infect **13**:90-99.

5. **Berthelot P., F. Grattard, A. Ros, F. Lucht, and B. Pozzetto**. 1998. Nosocomial legionellosis outbreak over a three-year period: investigation and control. Clin. Microbiol. Infect. **4**:385-391.

6. **Colbourne J. S., and P. J. Dennis**. 1985. Distribution and persistence of *Legionella* in water systems. Microbiol. Sci **2**:40-43.

7. **Colbourne J. S., P. J. Dennis, R. M. Trew, C. Berry, and G. Vesey**. 1988. *Legionella* and public water supplies. Water Science and Technology WSTED 4 **20**:5-10.

8. **Eichler S., M. G. Weinbauer, D. Dominik, and M. Höfle**. 2004. Extraction of total RNA and DNA from bacterioplankton, chapter 1.0.8, S. 103-120. *In* G.A.Kowalchuk; F.J.D.Bruijn; I.M.Head; A.D.L.Akkermans; and J.D.van Elsas (Hrsg.), Molecular microbial ecology manual, 2. Aufl. Kluwer Academic Publishers, Dordrecht, The Netherlands.

9. **Eichler S., R. Christen, C. Höltje, P. Westphal, J. Bötel, I. Brettar, A. Mehling, and M. G. Höfle**. 2006. Composition and Dynamics of Bacterial Communities of a Drinking Water Supply System as Assessed by RNA- and DNA-Based 16S rRNA Gene Fingerprinting. Appl. Environ. Microbiol. **72**:1858–1872.

10. **Exner M., and T. Kistemann**. 2004. Bedeutung der Verordnung über die Qualität von Wasser für den menschlichen Gebrauch (Trinkwasserverordnung 2001) für die Krankenhaushygiene. Bandesgesandheitsblatt - Gesandheitsforschung - Gesandheitsschutz **47**:384-391.

11. **Fields B. S., R. F. Benson, and R. E. Besser**. 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. Clin. Microbiol. Rev. **15**:506.

12. **Fry N. K., B. Afshar, P. Visca, D. Jonas, J. Duncan, E. Nebuloso, A. Anderwood, and T. G. Harrison**. 2005. Assessment of fluorescent amplified fragment length polymorphism analysis for epidemiological genotyping of *Legionella pneumophila* serogroup 1. Clin. Microbiol. Rev. **11**:704-712.

13. **Gaia V., N. K. Fry, B. Afshar, P. C. Luck, H. Meugnier, J. Etienne, R. Peduzzi, and T. G. Harrison**. 2005. Consensus sequence-based scheme for epidemiological typing of clinical and environmental isolates of *Legionella pneumophila*. J. Clin. Microbiol. **43**:2047.

14. **Glick T. H., M. B. Gregg, B. Berman, G. Mallison, W. W. Rhodes, and I. Kassanoff**. 1978. Pontiac fever. An epidemic of unknown etiology in a health department: I. Clinical and epidemiologic aspects. Am. J. Epidemiol **107**:149-160.

15. **Harth-Chu E., R. T. Espejo, R. Christen, C. A. Guzman, and M. G. Höfle**. 2009. Multiple-Locus Variable-Number Tandem-Repeat Analysis for Clonal Identification of *Vibrio parahaemolyticus* Isolates by Using Capillary Electrophoresis. Appl. Environ. Microbiol. **75**:4079-4088.

16. **Joseph C. A., and European Working Group for *Legionella* Infections**. 2004. Legionnaires' Disease in Europe 2000–2002. Epidemiol. Infect. **132**:417-424.

17. **Keim P., L. B. Price, A. M. Klevytska, K. L. Smith, J. M. Schupp, R. Okinaka, P. J. Jackson, and M. E. Hugh-Jones**. 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J. Bacteriol. **182**:2928-2936.

18. **Klevytska A. M., L. B. Price, J. M. Schupp, P. L. Worsham, J. Wong, and P. Keim**. 2001. Identification and Characterization of Variable-Number Tandem Repeats in the *Yersinia pestis* Genome. J. Clin. Microbiol. **39**:3179-3185.

19. **Le Flèche P., Y. Hauck, L. Onteniente, A. Prieur, F. Denoeud, V. Ramisse, P. Sylvestre, G. Benson, F. Ramisse, and G. Vergnaud**. A tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus anthracis*. BMC Microbiol **1**:2-2.

20. **Leoni E., G. De Luca, P. P. Legnani, R. Sacchetti, S. Stampi, and F. Zanetti**. 2005. *Legionella* waterline colonization: detection of *Legionella* species in domestic, hotel and hospital hot water systems. J. Appl. Microbiol. **98**:373–379.

21. **Lo Presti F., S. Riffard, H. Meugnier, M. Reyrolle, Y. Lasne, P. A. Grimont, F. Grimont, R. F. Benson, D. J. Brenner, A. G. Steigerwalt, J. Etienne, and J. Freney**. 2001. *Legionella gresilensis* sp. nov. and *Legionella beliardensis* sp. nov., isolated from water in France. Int. J. Syst. Evol. Microbiol **51**:1949-1957.

22. **Lo Presti F., S. Riffard, H. Meugnier, M. Reyrolle, Y. Lasne, P. A. Grimont, F. Grimont, F. Vandenesch, J. Etienne, J. Fleurette, and J. Freney**. 1999. *Legionella taurinensis* sp. nov., a new species antigenically similar to *Legionella spiritensis*. Int. J. Syst. Bacteriol **49 Pt 2**:397-403.

23. **Lück P. C., I. Leupold, M. Hlawitschka, J. H. Helbig, I. Carmienke, L. Jatzwauk, and T. Guderitz**. 1993. Prevalence of *Legionella* species, serogroups, and monoclonal subgroups in hot water systems in south-eastern Germany. Zentralbl Hyg Umweltmed **193**:450-460.

24. **Maiden M. C., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, and others**. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A **95**:3140.

25. **Mathys W., J. Stanke, M. Harmuth, and E. Junge-Mathys**. 2008. Occurrence of *Legionella* in hot water systems of single-family residences in suburbs of two German cities with special reference to solar and district heating. Int J Hyg Environ Health **211**:179-185.

26. **Nederbragt A. J., A. Balasingham, R. Sirevåg, H. Utkilen, K. S. Jakobsen, and M. J. Anderson-Glenna**. 2008. Multiple-locus variable-number tandem repeat analysis of *Legionella pneumophila* using multi-colored capillary electrophoresis. J. Microbiol. Methods **73**:111-117.

27. **Obrien S.** 1993. Legionnaires' disease: the infective dose paradox. The Lancet **342**:5-6.

28. **Paszko-Kolva C., M. Shahamat, and R. R. Colwell**. 1992. Long-term survival of *Legionella pneumophila* serogroup 1 ander low-nutrient conditions and associated morphological changes. FEMS Microbiol Lett **102**:45-55.

29. **Perola O., J. Kauppinen, J. Kusnetsov, J. Heikkinen, C. Jokinen, and M. L. Katila**. 2002. Nosocomial *Legionella pneumophila* serogroup 5 outbreak associated with persistent colonization of a hospital water system. Apmis **110**:863-868.

30. **Pourcel C., Y. Vidgop, F. Ramisse, G. Vergnaud, and C. Tram**. 2003. Characterization of a Tandem Repeat Polymorphism in *Legionella pneumophila* and Its Use for Genotyping. J. Clin. Microbiol. **41**:1819-1826.

31. **Pourcel C., P. Visca, B. Afshar, S. D'Arezzo, G. Vergnaud, and N. K. Fry**. 2007. Identification of Variable-Number Tandem-Repeat (VNTR) Sequences in *Legionella pneumophila* and Development of an Optimized Multiple-Locus VNTR Analysis Typing Scheme. J. Clin. Microbiol. **45**:1190-1199.

32. **Ratcliff R. M., J. A. Lanser, P. A. Manning, and M. W. Heuzenroeder**. 1998. Sequence-based classification scheme for the genus *Legionella* targeting the mip gene. J. Clin. Microbiol **36**:1560-1567.

33. **Ratzow S., V. Gaia, J. H. Helbig, N. K. Fry, and P. C. Luck**. 2007. Addition of neuA, the Gene Encoding N-Acylneuraminate Cytidylyl Transferase, Increases the Discriminatory Ability of the Consensus Sequence-Based Scheme for Typing *Legionella pneumophila* Serogroup 1 Strains. J. Clin. Microbiol. **45**:1965-1968.

34. **Roig J.** 2003. Legionnaires' disease: a rational approach to therapy. J. Antimicrob. Chemother. **51**:1119-1129.

35. **Samrakandi M. M., S. L. G. Cirillo, D. A. Ridenour, L. E. Bermudez, and J. D. Cirillo**. 2002. Genetic and Phenotypic Differences between *Legionella pneumophila* Strains. J. Clin. Microbiol. **40**:1352-1362.

36. **Steinert M., L. Emödy, R. Amann, and J. Hacker**. 1997. Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. Appl. Environ. Microbiol **63**:2047-2053.

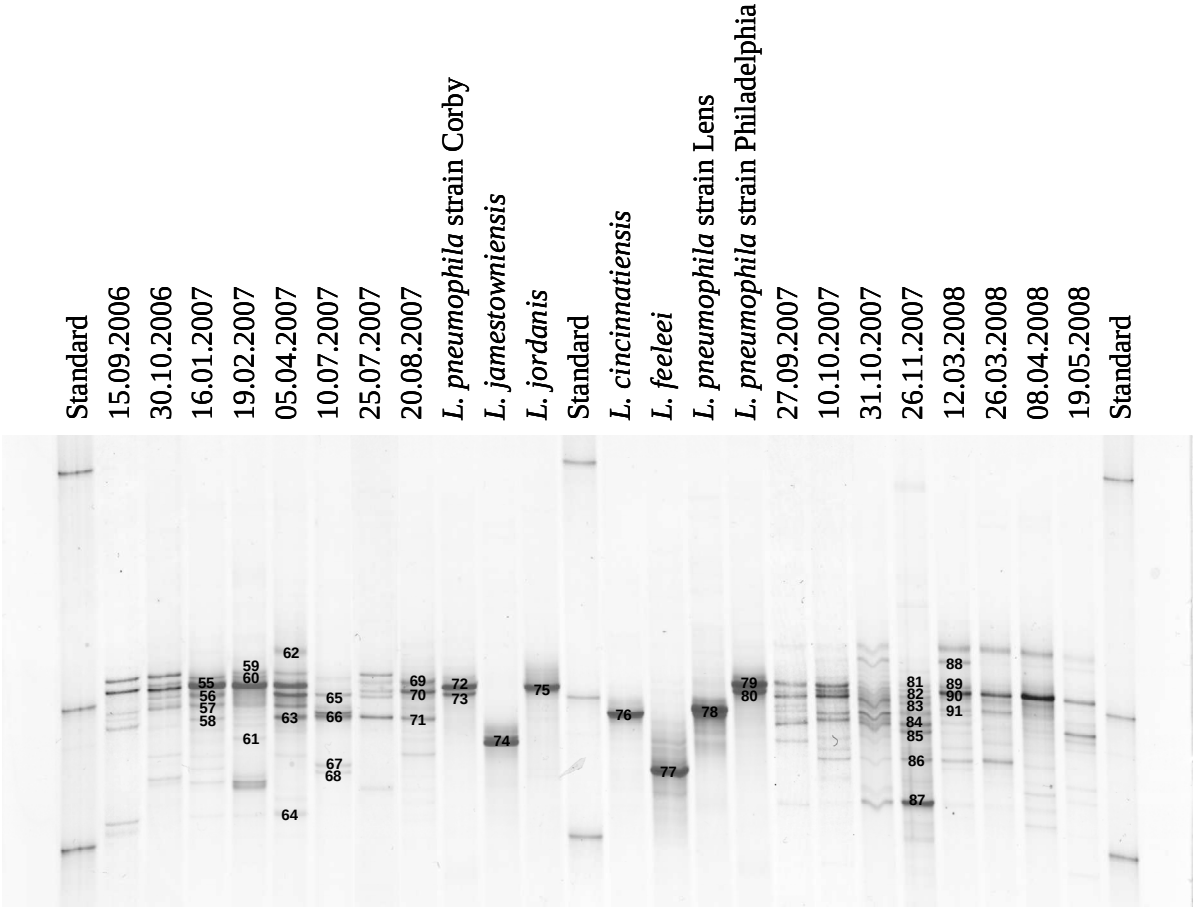
37. **Steinert M., U. Hentschel, and J. Hacker**. 2002. *Legionella pneumophila*: an aquatic microbe goes astray. FEMS Microbiol. Rev **26**:149-162.

38. **Tuntiwechapikul W., and M. Salazar**. 2002. Mechanism of in Vitro Expansion of Long DNA Repeats: Effect of Temperature, Repeat Length, Repeat Sequence, and DNA Polymerases. Biochemistry **41**:854-860.

39. **Weinbauer M. G., C. Beckmann, and M. G. Höfle**. 1998. Utility of Green Fluorescent Nucleic Acid Dyes and Aluminum Oxide Membrane Filters for Rapid Epifluorescence Enumeration of Soil and Sediment Bacteria. Appl. Environ. Microbiol. **64**:5000–5003.

40. **Yu V. L., J. F. Plouffe, M. C. Pastoris, J. E. Stout, M. Schousboe, A. Widmer, J. Summersgill, T. File, C. M. Heath, D. L. Paterson, and A. Chereshsky**. 2002. Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative survey. J. Infect. Dis. **186**:127-128.

5.8 Supplementary material



SUPPLEMENTARY FIGURE S1. Single-strand conformation polymorphism (SSCP) fingerprints of Legionella genus-specific screening. For drinking water samples DNA was extracted from frozen filters and genus-specific PCR was performed with primer pair “Lgsp17F” and “Lgsp28R” (s. material and methods). In the centre of the gel, single stranded PCR products from 7 reference strains are analysed. Standard, species standard of three different species for calculation of running distances. Numbers represent sequenced and identified bands with GenBank accession numbers GU598175-GU598211 given in supplementary Tab. 1.

SUPPLEMENTARY TABLE S1. Taxonomic identification of single 16S rRNA gene sequences obtained from SSCP fingerprints shown in Fig. 2 (band number 55-91).

Band number	SSCP band no.	GenBank Accession no.	Closest 16S rRNA gene sequence (accession no.)	% Similarity	Closest described species (Accession no.)	% Similarity
BN55	13-4-1	GU598175	Uncultured bacterium clone 2C229243 (EU800974.1)	99	*Legionella parisiensis* (AJ601375.1)	97
BN56	13-4-2	GU598176	Uncultured Legionella sp. clone Tang2-4 (AY924016.1)	98	Legionella pneumophila str. Corby (CP000675.2)	98
BN57	13-4-3	GU598177	Uncultured Legionella sp. clone S5-7 (AY924153.1)	96	Legionella pneumophila subsp. pascullei (AF122885.1)	96
BN58	13-4-4	GU598178	L.quateirensis (Z49732.1)	98	L.quateirensis (Z49732.1)	98
BN59	13-5-1	GU598179	Uncultured bacterium clone 2C229243 (EU800974.1)	100	L.quateirensis (Z49732.1)	97
BN60	13-5-2	GU598180	Uncultured Legionella sp. clone Tang2-4 (AY924016.1)	96	L.quateirensis (Z49732.1)	96
BN61	13-5-3	GU598181	Uncultured bacterium clone E-1956-31 (EU083443.1)	96	Legionella longbeachae strain ATCC 33462 (AY444740.1)	95
BN62	13-6-1	GU598182	Uncultured bacterium clone 1B17 (EU835422.1)	97	Legionella pneumophila subsp. pascullei (AF122885.1)	96
BN63	13-6-2	GU598183	Legionella pneumophila subsp. pascullei (AF122885.1)	96	Legionella pneumophila subsp. pascullei (AF122885.1)	96
BN64	13-6-3	GU598184	Legionella-like amoebal pathogen 8 (U64035.1)	96	Legionella-like amoebal pathogen 8 (U64035.1)	96
BN65	13-7-1	GU598185	Uncultured Legionella sp. clone Tang7-3 (AY924081.1)	97	L.quateirensis (Z49732.1)	97

BN66	13-7-2	GU598186	Uncultured Legionella sp. clone Tsw1-7 (AY923991.1)	97	Legionella pneumophila strain Alcoy 2300/99 (EU054324.1)	94
BN67	13-7-3	GU598187	Legionella waltersii strain 2074-AUS-E (NR_024969.1)	96	Legionella pneumophila strain Alcoy 2300/99 (EU054324.1)	95
BN68	13-7-4	GU598188	Uncultured bacterium clone WC3_79 (GQ264139.1)	96	Legionella waltersii strain 2074-AUS-E (NR_024969.1)	96
BN69	13-9-1	GU598189	Legionella pneumophila subsp. pascullei (AF122885.1)	95	Legionella pneumophila subsp. pascullei (AF122885.1)	95
BN70	13-9-2	GU598190	Uncultured bacterium clone 661199 (DQ404921.1)	94	Legionella impletisoli (AB233209.1)	93
BN71	13-9-3	GU598191	Uncultured Legionella sp. clone Tsw6-4 (AY924041.1)	99	Legionella dresdeniensis (AM747393.1)	96
BN72	13-10-1	GU598192	Legionella pneumophila str. Corby (CP000675.2)	99	Legionella pneumophila str. Corby (CP000675.2)	99
BN73	13-10-2	GU598193	Legionella pneumophila str. Corby (CP000675.2)	100	Legionella pneumophila str. Corby (CP000675.2)	100
BN74	13-11-1	GU598194	L.jamestowniensis (ATCC 35298) (X73409.1)	96	L.jamestowniensis (ATCC 35298) (X73409.1)	96
BN75	13-12-1	GU598195	L.jordansis (Z32667.1)	100	L.jordansis (Z32667.1)	100
BN76	13-14-1	GU598196	L.cincinatiensis (ATCC 43753) (X73407.1)	98	L.cincinatiensis (ATCC 43753) (X73407.1)	98
BN77	13-15-1	GU598197	L.feeleii sgp2 (ATCC 35849) (X73406.1)	100	L.feeleii sgp2 (ATCC 35849) (X73406.1)	100
BN78	13-16-1	GU598198	Legionella pneumophila str. Lens (CR628337.1)	100	Legionella pneumophila str. Lens (CR628337.1)	100

BN79	13-17-1	GU598199	Legionella pneumophila subsp. pneumophila str. Philadelphia 1 (AE017354.1)	100	Legionella pneumophila subsp. pneumophila str. Philadelphia 1 (AE017354.1)	100
BN80	13-17-2	GU598200	Legionella pneumophila subsp. pneumophila str. Philadelphia 1 (AE017354.1)	100	Legionella pneumophila subsp. pneumophila str. Philadelphia 1 (AE017354.1)	100
BN81	13-21-1	GU598201	Uncultured Legionella sp. clone Tsw8-6 (AY924060.1)	95	Legionella pneumophila subsp. pascullei (AF122885.1)	95
BN82	13-21-2	GU598202	Uncultured bacterium clone 255b2 (EF459912.1)	97	L.quateirensis (Z49732.1)	95
BN83	13-21-3	GU598203	Legionella-like amoebal pathogen 2 (U44909.1)	97	Legionella-like amoebal pathogen 2 (U44909.1)	97
BN84	13-21-6	GU598204	L.quateirensis (Z49732.1)	98	L.quateirensis (Z49732.1)	98
BN85	13-21-7	GU598205	Uncultured bacterium clone YSK16S-15 (EF612978.1)	97	Legionella pneumophila str. Lens (CR628337.1)	94
BN86	13-21-8	GU598206	Uncultured bacterium clone 1B17 (EU835422.1)	97	Legionella pneumophila subsp. pascullei (AF122885.1)	97
BN87	13-21-9	GU598207	Uncultured Legionella sp. clone S5-4 (AY924150.1)	95	Legionella dresdeniensis (AM747393.1)	94
BN88	13-22-2	GU598208	Uncultured Legionella sp. clone Tag3-4 (AY924175.1)	96	L.worsliensis (Z49739.1)	94
BN89	13-22-3	GU598209	Uncultured gamma proteobacterium clone SI-2F_G05 (EF221404.1)	98	Legionella pneumophila subsp. pneumophila str. Philadelphia 1 (AE017354.1)	97

BN90	13-22-4	GU598210	Uncultured Legionella sp. clone T0leg_14 (GQ861548.1)	96	Legionella pneumophila str. Corby (CP000675.2)	96
BN91	13-22-5	GU598211	Uncultured bacterium clone Roi_L1-H10-T7 (FN296944.1)	98	Legionella pneumophila subsp. pascullei (AF122885.1)	97

CHAPTER 6

General discussion

Leila Kahlisch

Dept. Vaccinology and Applied Microbiology
Helmholtz Center for Infection Research (HZI)
Inhoffenstrasse 7, 38124 Braunschweig, Germany

Chapter 6 General discussion

6.1 Microbial community, structure and composition as analyzed by molecular fingerprinting

We applied SSCP fingerprinting to assess the community structure of the drinking water microflora of a drinking water supply system (DWSS) in Northern Germany originating from two reservoirs in the Harz mountains (Chapter 4, (17)). During one and a half year (autumn 2006 until spring 2008), drinking water from the tap on the HZI campus was sampled and analyzed at monthly intervals to understand seasonal dynamics affecting the overall community structure. The analyses included SSCP fingerprinting, sequencing of relevant bands and phylogenetic assignment of the 16S rRNA sequences. The seasonal dynamics of the tap water was characterized by three constant and 40-80 varying phylotypes. The three major phylotypes, that were constantly present over the whole time period, belonged to uncultured bacterial species of the phylum *Actinobacteria* and the classes *Betaproteobacteria* and *Alphaproteobacteria*. Several other phylotypes occurred only during specific periods and can therefore be seen as indicators of changes in the structure and composition of the drinking water microflora. Some of the phylotypes have already been detected in the previous study on the respective DWSS (13). In this study, Eichler et al. (13) observed that the taxonomic composition of the bacterial communities from both reservoirs was very different at the species level. These differences were probably resulting from the different limnological conditions of the two reservoirs. Our detailed analysis of the seasonal community dynamics of the tap water confirmed that both source waters had a significant influence on the composition of the drinking water microflora (Chapter 4, (17)).

Using SSCP fingerprinting we were able to assess the relative abundances of all bacterial members of the drinking water microflora to a threshold of 0.1% relative abundance. By sequencing of the 408nt amplicon from the SSCP gels, we were able to identify the single members of the community at about the species level. We therefore compared the retrieved sequence information to international database entries. Since sequence information can be incorrectly deposited in databases, problems with a correct assignment can arise (12). We tried to overcome these problems by using different databases and also by comparing the retrieved sequences to our own data previously obtained on the same DWSS.

It is discussed, which part of the 16S rRNA gene is informative enough to allow taxonomic resolution up to the species level. Sequencing of the whole stretch is not always feasible and also time consuming if frequently applied. New sequencing technologies (e.g. pyrosequencing) are indeed faster but have the limitation of only producing short sequence reads. Therefore, one has to concentrate on the nine “hypervariable regions” (V1-V9) of the 16S rRNA gene that demonstrate considerable sequence diversity among different bacteria

(41). Sundquist et al. (39) studied the impact of read length on discriminatory power targeting the 16S rRNA gene in pyrosequencing. They also compared the utility of specific variable regions and came to the conclusion that the V6 region, which is only about 60 bp in length, is giving in about 50% of the cases a species level resolution (39). Although the V6 is the shortest hypervariable region, it provided the highest nucleotide diversity and discriminatory power for 110 bacterial species (Chakravorty et al. (10)). Since genetic profiling of bacterial communities is often based on PCR amplification, separation of 16S rRNA gene amplicons (like in our single strand conformation polymorphism (SSCP) analysis) is often limited by the product length that can be separated. Therefore, only parts of the 16S rRNA gene can be analyzed. Schmalenberger et al. compared different combinations of the variable regions (V2 and V3, V4 and V5 and V6 to V8) to discriminate 13 bacterial species by SSCP analysis (34). They observed that additional SSCP bands which can be caused by intraspecies operon heterogeneities or by more than one conformation of the same sequence are less if products contain the V4 to V5 region. Therefore, the authors stated that this stretch of the 16S rRNA gene seemed to be most suitable for a PCR-based microbial community analysis. In fact, this part of the 16S rRNA gene which is targeted by so called “universal” community primers (“com Primer”, position 519-926 in *E. coli* numbering (36)) was used in several studies to determine successfully the composition of aquatic microbial communities (19) and was therefore chosen for our analysis on the seasonal variation.

In contrast to Eichler et al. (13), we analyzed the community structure and composition by DNA-based fingerprinting for the seasonal dynamics. For the live/dead distinction (see Chapter 2 and 3), both, RNA and DNA based analyses were performed. According to Eichler et al. (13), the DNA-based fingerprints behave rather conservatively and demonstrate high source water dependence whereas the RNA-based fingerprints reveal changes that are caused by rather recent changes in the DWSS. The analysis via RNA-based fingerprints on the same set of samples will provide further insights into shifts in the community, that are for example caused by water processing such as chlorination. These analyses for the seasonal variation are part of another doctoral thesis and will not be discussed here. Major differences between DNA and RNA- based analyses on the same DWSS are discussed in this thesis in Chapter 2 and 3 and the respective discussion part 6.2.

In conclusion, the insights gained from this study and also from Eichler et al. (13) confirmed that molecular analysis based on fingerprints can be a valuable monitoring tool for drinking water supply systems especially when applied at different degrees of resolution.

6.2 The viable part of the bacterial community as assessed by fluorescent stains

The phylogenetic analysis of the live and dead fraction of the drinking water microflora was a main task of this thesis. We have performed a combined molecular-cellular approach, i.e. live/dead staining (Propidium Iodide (PI) and SYTO9), Fluorescence Activated Cell Sorting (FACS) and community fingerprinting followed by sequence analyses of the fingerprint bands (Chapter 2 and 3). On three sampling dates finished drinking water samples were stained with SYTO9 and PI and subsequently subjected to Fluorescence Activated Cell Sorting (FACS). The membrane intact ("live") and membrane injured cellular fractions ("dead") were separated and compared to the unsorted cells. Nucleic acids (DNA and RNA) were extracted from the three fractions, i.e. "unsorted", "live" and "dead", and analyzed by 16S rRNA-based and 16S rRNA gene-based SSCP fingerprinting followed by sequencing of the fingerprint bands to provide insight into the taxonomic composition of the bacterial community. We showed that i) DNA- and RNA-based overall community structure differed substantially, ii) the bacterial community retrieved from RNA and DNA reflected different bacterial species, i.e. phylotypes, (31 RNA-based phylotypes and 24 DNA-based phylotypes; only two common phylotypes), iii) the retrieved species were primarily of aquatic origin, and iv) the fraction of phylotypes showing only membrane injured cells, membrane intact cells and both was comparable for RNA- and DNA-based analyses.

Because we think that it is of major concern to estimate the viable part of a drinking water bacterial community, we chose to apply fluorescent dyes in combination with molecular methods for the separation of viable from dead cells. Membrane injury was chosen because it is considered by many studies as irreversible criterion for cell death (8, 18, 20). Staining with PI was shown to be a good estimate for membrane injury for *Bacteria* and *Archaea* (23). The combined staining procedure with SYTO9 and PI for distinction of membrane injured and intact bacteria was extensively compared with other methods and evaluated by many studies (14, 20). Besides the evaluation of methodological aspects, recent studies were done for drinking water with bacteria added to the indigenous microflora. Berney et al. (8) tested the use of SYTO9 and PI to assess the survival of *E. coli* in drinking water that was subjected to UV and sunlight irradiation. Compared to a set of different viability stains, the study showed, that loss of membrane integrity as indicated by SYTO9/PI staining was the final signal after decrease of all other tested physiological functions. In a later study, Berney et al. (7) used PI staining to analyze the bacterial microflora of a set of drinking water samples. The viable fraction of drinking water bacteria was higher for bottled water (about 90%) and drinking fountain water (about 85%) than for drinking water from the tap (about 66%). The high percentage of viable cells coincided with a high ATP content. The comparison of PI staining with other methods demonstrated PI staining was a valuable criterion for live-dead distinction for drinking water bacteria. Our percentage of viable cells for our drinking water system

(53%) therefore is in line with these results. Boulos et al. (9) reported the application of several dyes to drinking water and similar trends were found between SYTO9/PI staining and 5-cyano-2,3-ditolyl tetrazolium (CTC) counts in the absence of stress. In a microcosm experiments with coastal Mediterranean sea water, Gasol and colleagues compared viability as assessed by DNA content, SYTO9/PI staining and nucleoid-content (NuCC cells) (16). They found a similar fraction of “live” bacteria assessed by all three methods thus confirming the use of the SYTO9/PI dye system which is commercially available as “BacLight Kit”.

Additionally, some parameters (e.g. chlorination or water temperature) are known to have an effect on the staining procedure itself (9). Since our tap water is not containing measurable amounts of chlorine and we processed all samples immediately and at the same temperature, we think that these objections are of minor relevance for the analysis of our DWSS. Our drinking water community was dominated by phyla and classes typical for freshwater environments, i.e. *Bacteroidetes, Cyanobacteria, Betaproteobacteria* and *Gammaproteobacteria* (43). This was also the case when looking at the higher level of phylogenetic resolution, i.e. the phylotypes that were resolved at the species level. The majority of the phylotypes (76%) were most closely related to sequences retrieved from aquatic habitats. This is consistent with findings of the study of the whole drinking water supply system by Eichler et al. (13) four years ago. A comparison of the composition of the unsorted and sorted drinking water fractions additionally showed that we were able to recover most of the phylotypes after the sorting process and only few phylotypes were lost.

The sorting process via fluorescence activated cell sorting could contribute to a possible bias. We used parts of the drinking water concentrate that were only stained with one of the dyes to calibrate the machine via determining the relative intensities of the emission spectra of PI and SYTO9. Since this calibration step was done before every sorting procedure and depends highly on the operational settings that are performed by the operator, minor changes between the three sorting processes can not be excluded. We tried to overcome this problem by always using the same settings and programs and the calibration and sorting was always performed by the same experienced operator.

It was shown in a study by Stocks (38) that it was possible, especially in the presence of excess DNA, to obtain DNA labelled simultaneously by SYTO9 and PI. The author concluded that the choice of dye concentrations in determining bacterial viability is of high importance. Moreover it has to be ensured that PI is present in excess whereas the concentration of SYTO9 is not critical (38). We followed the specifications of the manufacturer very precisely in order to prevent problems with the live-dead distinction.

Since we used appropriate controls, we think that the staining procedure is rather robust and can be used for the sorting of drinking water bacteria. Combined with the

adequate molecular analyses it can serve as a tool to investigate any targeted group of microorganisms, especially pathogens.

6.3 Molecular fingerprints for the detection and genotyping of *Legionella pneumophila* in drinking water

Legionella species constitute a group of bacteria that was recently discovered as emerging pathogens (40). Because they are ubiquitous in aquatic environments it is nearly impossible to prevent their entry into man-made aquatic systems (e.g. drinking water supply systems) where they occur in bulk water as well as in biofilms, preferably in the hot water distribution system (4, 35, 37). Thus, species of the genus *Legionella* can pose a significant health threat if present as the most infectious species and serotype, i.e. *Legionella pneumophila* serogroup 1.

The second major objective of this thesis was the quantification and high-resolution genotyping of *L. pneumophila* in drinking water. We therefore determined the presence of the genus *Legionella*, identified the present species and, if *Legionella pneumophila* was in the sample also tried to identify the genotype of the strain without cultivation. In the first part of the hierarchical approach, we used a genus-specific primer pair for the PCR detection of species of the genus *Legionella* in our DWSS. Most of our tap water samples yielded a positive screening result. We separated the PCR products using SSCP gel electrophoresis and sequenced most bands in order to obtain information about the present species. Most of the retrieved sequences revealed uncultured species of the genus *Legionella*. A few sequences could be clearly (over 98% sequence similarity) assigned to cultured species, and even *L. pneumophila* was detected in several samples (strain Philadelphia and strain Corby).

We investigated how abundant these pathogenic *Legionella* species are in our drinking water and therefore developed and applied a real-time based PCR quantification for *L. pneumophila*. The quantification revealed that *L. pneumophila* was present in countable numbers (90 cells/litre of finished drinking water) in two cold and three hot water sources of our DWSS. Although the dose of legionellae necessary for an infection of humans is unknown (WHO), it is assumed to be very low for susceptible persons. The infection dose highly depends on several parameters, e.g. number of legionellae in the water sample, effectiveness of dissemination through air via water droplets, host factors (advanced age, tobacco smoke, immunodeficiency) and also the virulence of the particular strain (6). In a study of Cirillo et al. (11), the invasive ability of *Legionella pneumophila* grown under standard conditions was compared with that of bacteria grown in *Acanthamoeba castellanii*, which represents a protozoan species serving as a natural host in the environment. The experiment showed that cells grown in amoeba were at least 100 fold more invasive to epithelial cells and 10 fold more invasive to macrophages (11). This association with

protozoa can be seen as a major factor for the ubiquitous presence of legionellae in the environment due to the fact that protozoa can protect the bacteria from adverse conditions and serve as a reservoir. Adequate treatment measures therefore should not only aim at killing the bacteria (e.g. chlorination, UV irradiation) but should also inhibit the survival of the protozoan host.

We determined the genotypes of *L. pneumophila* strains isolated from hot and cold water sources of our institute's distribution system. We used the method previously described by Nederbragt et al. (28) who transferred the MLVA typing scheme that was set up by Pourcel et al. (31, 32) onto capillary electrophoresis. Using the eight polymorphic tandem repeats comprising approach we could assign our isolates to three different genotypes. Since we had found a variety of different *Legionella* species by the genus specific screening and legionellae are not easy to cultivate, we assumed that more *L. pneumophila* genotypes could be present in our drinking water samples. Therefore, we applied the single primer sets from the MLVA-8 analysis to amplify the VNTR loci directly from the DNA extracted from the environmental sample. We succeeded in amplifying the different loci and separated the amplicons on SSCP gels (Chapter 5). For the first time, this approach enabled not only the detection of different *L. pneumophila* genotypes in environmental samples but also the possibility for sequencing of the VNTR products from the SSCP gel. By using SSCP analysis and sequencing, we were able to detect more *L. pneumophila* MLVA genotypes in drinking water samples than we could detect by isolation attempts in the same sample.

Some authors might argue that MLVA based analyses are not suitable for broad phylogenetic analyses due to high mutation rates and therefore corresponding values of homoplasies (1, 2). Significant differences in locus repeat units can even occur after several rounds of cultivation of the outbreak strain. But although tandem repeats have an enhanced inherent variability, it was shown in a study of *Staphylococcus aureus* isolates, that clusters of strains remained traceable when compared to AFLP data (26). Additionally, MLVA has turned out be very helpful for diagnostic and epidemiological typing of several other important pathogens (3, 15, 24, 27). For several genetically homogenous species like *Bacillus anthracis, Yersinia pestis* or *Mycobacterium tuberculosis*, MLVA can be regarded as the reference method (21, 22, 30). We therefore think that MLVA is an appropriate method for the genotyping of *L. pneumophila* strains. Additionally, our nucleic acid based diagnostic approach provides a higher level of resolution than the conventional typing by identification of serogroups.

6.4 Outlook

In the first part of the thesis, we were able to characterize the viable and dead part of the bacterial drinking water community with the help of fluorescent dyes. We therefore used a combination of SYTO9/PI staining, FACS sorting and molecular analyses of the sorted and unsorted fractions. To evaluate the use of this specific dye combination, targeting membrane integrity, it would be useful to compare the results also to other methods assessing viability of microorganisms. For example, viability PCR (EMA-PCR, (33)) could be used to assess viable bacteria in a drinking water sample. This relatively new method could provide some advantages. The concentration procedure necessary for the sorting process is not needed for this approach and since PCR is directly performed on the environmental sample, contamination is not a critical issue. Nevertheless, since ethidium monoazide can be actively exported from some bacterial cells (29), the implementation of this dye should be carefully evaluated for drinking water samples containing a variety of different species. In this regard, the use of Propidium monoazide (PMA) instead of EMA should be considered as a further development, because it is known to be more selective for penetrating only dead bacterial cells (29).

After we developed the combined approach of staining followed by molecular analyses, we applied it to a set of drinking water samples from three different dates. It would be of interest to characterize the viable members of the drinking water microflora with respect to many issues such as changes throughout a year, in the hot water system or after pressure loss events in the distribution system. The approach opens the possibility to analyze samples in a broader scope of application such as food or other water samples (e.g. bottled water, water from storage tanks, etc.). The knowledge about which bacterial species are viable in a given sample could substantially help to improve treatment processes and gives the possibility to assess potential risks. Since the developed procedure is rather time consuming and laborious it can presumably not be used as a tool for routine drinking water surveillance but can be implemented on a time to time basis to get a more detailed view on which bacterial species are alive and which are dead in our drinking water. This is of general relevance regarding healthy people with a good immune status but of special interest regarding immunocompromised persons that are more susceptible to infections caused by potentially pathogenic bacteria.

In the second part of the thesis we developed and adapted a nested hierarchical approach to detect, quantify and genotype *Legionella* species directly from environmental samples. Since we were able to detect by this *in-situ* approach more *L. pneumophila* genotypes per drinking water sample than by isolation efforts, it looks very promising to apply the method on a broader scale with drinking water and environmental samples to study the

molecular epidemiology of *L. pneumophila*. Several samples can be processed simultaneously, giving the possibility to analyze a larger set of samples, for example water samples collected from a seasonal cycle of a cooling tower. Especially for the hot water system, where we detected the highest numbers of *L. pneumophila* with real-time based quantification, the *L. pneumophila* populations could now be analyzed retrospectively because samples have been taken on a monthly basis and are stored frozen until further analysis. It would also be interesting to apply this universal tool to clinical samples. Since the isolation of *Legionella* species from environmental and clinical samples is challenging, the detection and genotyping of *L. pneumophila* by this molecular approach will be facilitated in general. For example, DNA can be extracted from sputum or lavage fluids of patients and directly analyzed by this approach enabling molecular epidemiology without the need for laborious isolation procedures. Samples from biobanks, where biological material of thousands of people is stored frozen, can be analyzed long time after the patient is cured. In general, this new method is also transferable to the typing of other pathogens of interest and therefore presents a promising new tool for molecular epidemiology of pathogens *in situ*.

In the next few years, progress due to new sequencing techniques (e.g. pyrosequencing technology) will be made in the field of molecular detection (25). This will provide further opportunities to develop tools for molecular detection and quantification of pathogenic bacteria. Recently, new results of complete sequencing of microorganisms of interest or specific samples (e.g. the human oral microbiome) appear on a daily basis and it is just a question of cost and time until sequencing of complex samples like drinking water will be achieved (5, 42). Since the complete sequencing of every single bacterium of a complex sample will not be achievable in the next few years (e.g. for a single patient), VNTR and MLVA analysis still provide good tools for the genotyping of bacterial species. Conducted as an *in situ* approach, like in our developed application, a universal tool for fast and low cost epidemiological analysis of bacterial pathogens is provided.

Taken together, our data suggest the suitability of molecular fingerprint techniques for genotyping of *L. pneumophila* isolates and open new avenues and vistas for epidemiological analyses of *L. pneumophila* infections. Therefore, this work confirms the importance of the development and application of molecular tools to supplement or even replace culture dependent methods for the detection, quantification and characterization of pathogens in environmental samples.

6.5 References

1. **Achtman M.** 2008. Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. Annu. Rev. Microbiol. **62**:53-70.

2. **Achtman M., G. Morelli, P. Zhu, T. Wirth, I. Diehl, B. Kusecek, A. J. Vogler, D. M. Wagner, C. J. Allender, W. R. Easterday, V. Chenal-Francisque, P. Worsham, N. R. Thomson, J. Parkhill, L. E. Lindler, E. Carniel, and P. Keim**. 2004. Microevolution and history of the plague bacillus, *Yersinia pestis*. Proc Natl Acad Sci U S A **101**:17837-17842.

3. **Al Dahouk S., P. L. Flèche, K. Nöckler, I. Jacques, M. Grayon, H. C. Scholz, H. Tomaso, G. Vergnaud, and H. Neubauer**. 2007. Evaluation of *Brucella* MLVA typing for human brucellosis. J Microbiol Methods **69**:137-145.

4. **Atlas R. M.** 1999. *Legionella*: from environmental habitats to disease pathology, detection and control. Environ. Microbiol **1**:283-293.

5. **Aury J., C. Cruaud, V. Barbe, O. Rogier, S. Mangenot, G. Samson, J. Poulain, V. Anthouard, C. Scarpelli, F. Artiguenave, and P. Wincker**. 2008. High quality draft sequences for prokaryotic genomes using a mix of new sequencing technologies. BMC Genomics **9**:603.

6. **Azara A., A. Piana, G. Sotgiu, M. Dettori, M. Deriu, M. Masia, B. Are, and E. Muresu**. 2006. Prevalence study of *Legionella* spp. contamination in ferries and cruise ships. BMC Public Health **6**:100.

7. **Berney M., M. Vital, I. Hülshoff, H. Weilenmann, T. Egli, and F. Hammes**. 2008. Rapid, cultivation-independent assessment of microbial viability in drinking water. Water Res **42**:4010-4018.

8. **Berney M., H. U. Weilenmann, and T. Egli**. 2006. Flow-cytometric study of vital cellular functions in *Escherichia coli* during solar disinfection (SODIS). Microbiology **152**:1719-1729.

9. **Boulos L., M. Prevost, B. Barbeau, J. Coallier, and R. Desjardins**. 1999. LIVE/DEAD BacLight : application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J Microbiol Methods **37**:77-86.

10. **Chakravorty S., D. Helb, M. Burday, N. Connell, and D. Alland**. 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods **69**:330-339.

11. **Cirillo J. D., S. Falkow, and L. S. Tompkins**. 1994. Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. Infect. Immun. **62**:3254-3261.

12. **Clarridge J. E.** 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev **17**:840-862.

13. **Eichler S., R. Christen, C. Höltje, P. Westphal, J. Bötel, I. Brettar, A. Mehling, and M. G. Höfle**. 2006. Composition and Dynamics of Bacterial Communities of a Drinking Water Supply System as Assessed by RNA- and DNA-Based 16S rRNA Gene Fingerprinting. Appl. Environ. Microbiol. **72**:1858–1872.

14. **Falcioni T., S. Papa, and J. M. Gasol**. 2008. Evaluating the flow-cytometric nucleic acid double-staining protocol in realistic situations of planktonic bacterial death. Appl Environ Microbiol **74**:1767-1779.

15. **Farlow J., D. M. Wagner, M. Dukerich, M. Stanley, M. Chu, K. Kubota, J. Petersen, and P. Keim**. 2005. *Francisella tularensis* in the United States. Emerg Infect Dis **11**:1835–1841.

16. **Gasol J., U. Zweifel, F. Peters, J. Fuhrman, and A. Hagstrom**. 1999. Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. Appl Environ Microbiol **65**:4475-4483.

17. **Henne K., L. Kahlisch, J. Draheim, I. Brettar, and M. G. Höfle**. 2008. Polyvalent fingerprint based molecular surveillance methods for drinking water supply systems. Water Science & Technology: Water Supply **8**:527-532.

18. **Hoefel D., P. Monis, W. Grooby, S. Andrews, and C. Saint**. 2005. Profiling bacterial survival through a water treatment process and subsequent distribution system. J Appl Microbiol **99**:175-186.

19. **Höfle M. G., S. Flavier, R. Christen, J. Bötel, M. Labrenz, and I. Brettar**. 2005. Retrieval of nearly complete 16S rRNA gene sequences from environmental DNA following 16S rRNA-based community fingerprinting. Environ Microbiol **7**:670-675.

20. **Joux F., and P. Lebaron**. 2000. Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. Microbes Infect **2**:1523-1535.

21. **Le Flèche P., M. Fabre, F. Denoeud, J. L. Koeck, and G. Vergnaud**. 2002. High resolution, on-line identification of strains from the *Mycobacterium tuberculosis* complex based on tandem repeat typing. BMC microbiology **2**:37.

22. **Le Flèche P., Y. Hauck, L. Onteniente, A. Prieur, F. Denoeud, V. Ramisse, P. Sylvestre, G. Benson, F. Ramisse, and G. Vergnaud**. A tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus anthracis*. BMC Microbiol **1**:2.

23. **Leuko S., A. Legat, S. Fendrihan, and H. Stan-Lotter**. 2004. Evaluation of the LIVE/DEAD BacLight Kit for Detection of Extremophilic Archaea and Visualization of Microorganisms in Environmental Hypersaline Samples. Appl. Environ. Microbiol. **70**:6884-6886.

24. **Lindstedt B., E. Heir, E. Gjernes, T. Vardand, and G. Kapperud**. 2003. DNA fingerprinting of Shiga-toxin producing *Escherichia coli* O157 based on Multiple-Locus Variable-Number Tandem-Repeats Analysis (MLVA). Ann Clin Microbiol Antimicrob **2**:12.

25. **Mardis E. R.** 2008. The impact of next-generation sequencing technology on genetics. Trends Genet **24**:133-141.

26. **Melles D., L. Schouls, P. François, S. Herzig, H. Verbrugh, A. van Belkum, and J. Schrenzel**. 2009. High-throughput typing of *Staphylococcus aureus* by amplified fragment length polymorphism (AFLP) or multi-locus variable number of tandem repeat analysis (MLVA) reveals consistent strain relatedness. Eur J Clin Microbiol Infect Dis **28**:39-45.

27. **Murphy M., D. Corcoran, J. F. Buckley, M. O'Mahony, P. Whyte, and S. Fanning**. 2007. Development and application of Multiple-Locus Variable Number of tandem repeat Analysis (MLVA) to subtype a collection of *Listeria monocytogenes*. Int J Food Microbiol **115**:187-194.

28. **Nederbragt A. J., A. Balasingham, R. Sirevåg, H. Utkilen, K. S. Jakobsen, and M. J. Anderson-Glenna**. 2008. Multiple-locus variable-number tandem repeat analysis of *Legionella pneumophila* using multi-colored capillary electrophoresis. J. Microbiol. Methods **73**:111-117.

29. **Nocker A., C. Cheung, and A. K. Camper**. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods **67**:310-320.

30. **Pourcel C., F. Andre-Mazeaud, H. Neubauer, F. Ramisse, and G. Vergnaud**. 2004. Tandem repeats analysis for the high resolution phylogenetic analysis of *Yersinia pestis*. BMC microbiology **4**:22.

31. **Pourcel C., Y. Vidgop, F. Ramisse, G. Vergnaud, and C. Tram**. 2003. Characterization of a Tandem Repeat Polymorphism in *Legionella pneumophila* and Its Use for Genotyping. J. Clin. Microbiol. **41**:1819-1826.

32. **Pourcel C., P. Visca, B. Afshar, S. D'Arezzo, G. Vergnaud, and N. K. Fry**. 2007. Identification of Variable-Number Tandem-Repeat (VNTR) Sequences in *Legionella pneumophila* and Development of an Optimized Multiple-Locus VNTR Analysis Typing Scheme. J. Clin. Microbiol. **45**:1190-1199.

33. **Rudi K., B. Moen, S. M. Dromtorp, and A. L. Holck**. 2005. Use of Ethidium Monoazide and PCR in Combination for Quantification of Viable and Dead Cells in Complex Samples. Appl. Environ. Microbiol. **71**:1018-1024.

34. **Schmalenberger A., F. Schwieger, and C. C. Tebbe**. 2001. Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. Appl Environ Microbiol **67**:3557-3563.

35. **Schwartz T., S. Hoffmann, and U. Obst**. 1998. Formation and bacterial composition of young, natural biofilms obtained from public bank-filtered drinking water systems. Water Res **32**:2787-2797.

36. **Schwieger F., and C. C. Tebbe**. 1998. A New Approach To Utilize PCR-Single-Strand-Conformation Polymorphism for 16S rRNA Gene-Based Microbial Community Analysis. Appl. Environ. Microbiol. **64**:4870-4876.

37. **Steinert M., U. Hentschel, and J. Hacker**. 2002. *Legionella pneumophila*: an aquatic microbe goes astray. FEMS Microbiol Rev **26**:149-162.

38. **Stocks S. M.** 2004. Mechanism and use of the commercially available viability stain, BacLight. Cytometry A **61**:189-195.

39. **Sandquist A., S. Bigdeli, R. Jalili, M. Druzin, S. Waller, K. Pullen, Y. El-Sayed, M. M. Taslimi, S. Batzoglou, and M. Ronaghi**. 2007. Bacterial flora-typing with targeted, chip-based Pyrosequencing. BMC Microbiol **7**:108.

40. **Szewzyk U., R. Szewzyk, W. Manz, and K. H. Schleifer**. 2000. Microbiological safety of drinking water. Annu. Rev. Microbiol **54**:81-127.

41. **Van de Peer Y., S. Chapelle, and R. De Wachter**. 1996. A quantitative map of nucleotide substitution rates in bacterial rRNA. Nucleic Acids Res **24**:3381-3391.

42. **Zaura E., B. J. F. Keijser, S. M. Huse, and W. Crielaard**. 2009. Defining the healthy "core microbiome" of oral microbial communities. BMC Microbiol **9**:259.

43. **Zwart G., B. C. Crump, M. P. Kamst-van Agterveld, F. Hagen, and S. K. Han**. 2002. Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquat Microb Ecol **28**:141–155.